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MDavis

Signature

Feb 12, 2008

"It is the mark of an educated mind to entertain a thought without accepting it."

~Aristotle

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Temporal and spatial regulation of gene expression in *Drosophila melanogaster* development and immunity

by

Monica Marie Davis ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Molecular Biology and Genetics

Department of Biological Sciences

Edmonton, Alberta
Spring 2008

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Temporal and spatial regulation of gene expression in *Drosophila melanogaster* development and immunity** submitted by **Monica Marie Davis** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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FEB 11, 2008

To my mom, who taught me to be strong and independent and to think for myself. Her true strength is that she never held it against me when I used these virtues against her.

Abstract

I have used *Drosophila melanogaster* to study how systemic signals elicit temporally precise protein- and gene-specific responses during development and the immune response. First, I examined the hormonal regulation of tyrosine hydroxylase (TH), encoded by *pale* (*ple*), and its role in the maturation of the adult cuticle. Second, I explored the role of Dopa decarboxylase (DDC), encoded by *Ddc*, in the response to septic injury. Both TH and DDC are required for the production of dopamine (DA). Metabolites of DA are required for the synthesis of melanin and sclerotin during cuticle maturation, and the cytotoxic reactive quinones that are intermediates in the production of these compounds may play a role in the immune defence system.

A neuropeptide hormone cascade controls events surrounding eclosion in Drosophila. Together, ecdysis-triggering hormone, eclosion hormone, and crustacean cardioactive peptide (CCAP) control ecdysis, while Bursicon regulates post-eclosion events. I examined how these neuropeptides regulate the precise onset of adult cuticular tanning following eclosion. The *ple* transcript accumulates prior to eclosion, and levels remain high until well after eclosion, which suggests that transcription does not control the rapid onset of tanning that follows eclosion. TH protein and activity levels drop prior to eclosion and rise thereafter, suggesting that translational and possibly post-translational controls were operating. I showed that CCAP is necessary for the accumulation of TH following eclosion. In addition, TH is transiently activated by phosphorylation after eclosion as a result of Bursicon signalling. Therefore, the precise onset of tanning is regulated by CCAP control of TH translation and Bursicon activation of TH phosphorylation.

To understand how a systemic infection can induce tissue-specific expression of immune response genes, I analyzed the induction of *Ddc* following septic injury. The Drosophila immune response is induced primarily by the Toll and IMD pathways. IMD signalling also activates the Jun kinase (JNK) signalling pathway, which elicits its effects through the transcription factor AP-1. I found that *Ddc* is induced throughout the epidermis, not specifically at the site of injury, following infection. *Ddc* transcripts appear two hours after septic injury, and remain high for nine hours following gram

negative infection and 36 hours after gram positive infection. This induction requires a conserved AP-1 binding site upstream of the *Ddc* transcription start site. However, the Toll, IMD and JNK signalling pathways are dispensable for *Ddc* inducibility; instead, a novel p38 mitogen-activated protein kinase, p38c, is necessary for *Ddc* immune induction. A transcription factor, activated by p38c signalling, which recognizes the AP-1 binding site, remains to be identified. I propose that *Ddc* is induced in the epidermis to produce reactive quinones that have antimicrobial effects.

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List of abbreviations

aaNAT	Arylalkylamine N-acyltransferases
ecdysone	20-hydroxyecdysone
ATF2	Activating transcription factor 2
APF	after puparium formation
APH	Alkaline phosphatase
Aph	*alkaline phosphatase*
AMP	antimicrobial peptides
BIR	baculovirus IAP repeat
BSK	Basket
bsk	*basket*
b	*black*
BR	Broad
8-Br-cAMP	8-bromo-cyclic adenosine mono-phosphate
burs	*bursicon*
cAMP	cyclic adenosine mono-phosphate
CCAP-KO	CCAP cell knock-out flies
CNS	central nervous system
CCAP	Crustacean cardioactive peptide
CRC	Cryptocephal
crc	*cryptocephal*
cAMP	cyclic AMP
DD	death domain
DED	death effector domain
DNR1	Defence repressor 1
Dhpr	*Dihydropteridine reductase*
Dopa	dihydroxyphenylalanine
DN	dominant negative
DDC	Dopa decarboxylase

Ddc	*Dopa decarboxylase*
DA	dopamine
DL	Dorsal
DIF	Dorsal-related immunity factor
DVP	double vertical plates
eth	*ecdysis-triggering hormone*
ETH	Ecdysis-triggering hormone
EcR	ecdysone receptor
EcRE	ecdysone response element
eh	*eclosion hormone*
EH	Eclosion hormone
EH-KO	EH cell knock-out flies
ethr	*eth receptor*
ETHR	ETH receptor
EST	expressed sequence tag
ERK	extracellular signal-regulated kinase
dFADD	Fas-associate protein with death domain
fmr1	*fragile X mental retardation protein 1*
FMR1	fragile X mental retardation protein 1
GNBP	gram negative binding protein
GFP	green fluorescent protein
hs	heat shock
hep	*hemipterous*
HEP	Hemipterous
hn	*henna*
Imd	Immune deficiency
ird	*immune response defective*
IAP2	inhibitor of apoptosis protein 2
JNK	jun kinase
lic	*licorne*
Lic	Licorne

LPS	lipopolysaccharide
Lys	lysine
MP	Melanisation protease
DAP	meso-diaminopimelic acid
mRNA	messenger RNA
miRNA	microRNA
MAPK	mitogen-activated kinase
MAPKK	mitogen-activated kinase kinase
MAPKKK	mitogen-activated kinase kinase kinase
MyoD88	myeloid-differentiation factor 88
MIPs	myoinhibitory peptides
NADA	N-acetyl dopamine
N	nonenzymatic
NBAD	N-β-alanyl dopamine
O	other
ple	*pale*
pburs	*partner of bursicon*
PRR	pattern recognition receptors
PGN	peptidoglycan
PGRP	peptidoglycan recognition proteins
PSH	Persephone
PO	Phenol oxidase
PAH	Phenylalanine hydroxylase
POSH	Plenty of SH3s
PCR	polymerase chain reaction
PPO	Prophenoloxidase
PKA	Protein kinase A
RING	really interesting new gene
rpr	*reaper*
REL	Relish
RT	Reverse transcriptase

RT-PCR	Reverse transcriptase polymerase chain reaction
RpL32	*ribosomal protein 32*
RK	Rickets
rk	*rickets*
RISC	RNA-induced silencing complex
rl	*rolled*
Ser	Serine
SP	Serine protease
SPE	Serine protease E
TAB2	TAK1 binding protein 2
BH_4	tetrahydrobiopterin
TAK1	TGFβ-activated kinase 1
Tl	Toll
TIR	Toll/IL-2 receptor
TF	transcription factor
TGF	transforming growth factor
TPH	Tryptophan and phenylalanine hydroxylase
TNF	tumour necrosis factor
Tyr	tyrosine
TH	Tyrosine hydroxylase
Tyr-P	tyrosine-*O*-phosphate
USP	Ultraspiracle
UTR	untranslated region
WPP	white pre-pupae
WT	wild-type

Chapter 1: General Introduction

Temporal and spatial regulation of gene expression is at the basis of how organisms respond to developmental or external cues. Of particular interest is the widespread observation that systemic signals can be converted into appropriate cell-, protein-, or gene-specific responses. Hormones are a good example of a developmental cue that can elicit these distinct responses. In general, hormones are released into the blood stream, and this systemic release can induce a variety of responses in target cell populations. Often, an initial hormone signal is refined by the release of additional hormones. Perhaps the best studied invertebrate hormone is the steroid hormone ecdysone. It has known roles at all stages of *Drosophila melanogaster* development, and has become a favourite model system for steroid hormone signalling. Ecdysone triggers the activation of cell-specific gene cascades that ultimately lead to the production of a small set of downstream genes. Simultaneously, ecdysone can cause the synthesis and release of neuropeptide hormones that further regulate gene activity in the target cells.

A second example of systemic signalling that elicits cell- and gene-specific responses is immunity. A systemic signal is generated by components of invading microorganisms within the blood stream. Rapid cell- and gene-specific responses are elicited depending on the signalling pathway that is activated. The innate immune response is the first to be triggered in mammals following infection, and the only type of immunity in many organisms. Innate immunity has been most thoroughly studied in Drosophila, where it leads to the differential induction of multiple signalling pathways, each of which results in a temporally controlled, tissue-specific expression of a subset of immune response genes.

In this thesis, I chose to study the regulation of two genes, each of which participates in a temporally precise process that was set in motion by a systemic signal. One signal originated during normal development and the other during an innate immune response. I use Drosophila as the model organism to study these processes due to the advanced molecular and genetic techniques that are available. In the first half of this thesis, I analyze how neuropeptides regulate the precise timing of post-eclosion development. In the second half of my thesis, I investigate how microbes can induce tissue-specific temporal expression of immune response genes. The two areas of

experimentation are linked by the central theme of regulation of genes that are required for the epidermal production of dopamine. The first project involves the hormonal regulation of tyrosine hydroxylase activity and the second deals with the immune induction of *Dopa decarboxylase* transcription. Thus, my introduction is presented in two parts; the first deals with hormone signalling, the second with the innate immune response.

Part 1: Hormonal regulation of Drosophila development

The neuropeptide prothoracicotrophic hormone induces secretion of α-ecdysone from the prothoracic gland into the haemolymph (1), which is modified by peripheral tissues to produce the physiologically active form of the hormone, 20-hydroxyecdysone (hereafter referred to as ecdysone). Ecdysone controls every major developmental transition in Drosophila and is released prior to hatching, both larval moults, pupation and eclosion (Figure 1.1A). When ecdysone is not produced, the organism fails to transition to the next stage in development and dies (2,3).

The ecdysone receptor

The ecdysone receptor, which is a member of the nuclear hormone receptor superfamily (4), regulates gene transcription by binding ecdysone response elements (EcREs) following association with ecdysone. This ligand-dependent receptor is a heterodimer composed of one Ecdysone Receptor (EcR) and one Ultraspiracle (USP) subunit (5-8). USP is a homolog of the mammalian retinoic-X-receptor (9), and larvae mutant for the *usp* gene fail to moult and die late in the first instar (10). The mammalian homolog of EcR is the farnesoid-X-receptor (4). The *EcR* transcript is alternatively spliced to encode three receptor subtypes, EcR-A, EcR-B1, and EcR-B2 (Figure 1.1B) (11). These isoforms have different amino-termini, but share a common carboxy-terminal region containing a DNA-binding domain and a ligand-binding domain (11,12). Many studies have focused on defining temporal and spatial expression patterns of each EcR isoform through use of isoform-specific antibodies (11,13-16). EcR-A is expressed in the imaginal discs and imaginal rings that ultimately result in the formation of adult structures (11). In addition, EcR-A is expressed in the prothoracic gland cells (11,17) and type II larval neurons of the central nervous system (15), two tissues that have late larval functions. EcR-B1 is expressed in larval-specific tissues and those that form the adult

abdominal epithelium and midgut, called imaginal histoblasts (11). EcR-A is required for neuronal maturation, while EcR-B1 expression is correlated to neuronal regression (16), demonstrating the opposing functions of these isoforms. The specific expression profile of EcR-B2 has not yet been determined, due to a lack of a specific antibody to this isoform, which only has 17 unique amino acids that it does not share with EcR-B1. However, by expressing a dominant negative form of EcR to disrupt its function and then reintroducing only EcR-B2, a role for EcR-B2 in border-cell migration in the egg chamber, shortening in wandering larvae, and cuticular tanning at pupariation was demonstrated (18).

Four types of *EcR* mutants have been created. The first are *EcR* null mutants, which cannot produce any of the three isoforms, and die late in embryogenesis. The second type of mutation disrupts production of functional EcR-B1 and EcR-B2, but retains EcR-A function. These mutants rarely survive to the third instar and show defects in moulting and neuronal remodelling (19). The third class of mutation blocks expression of the EcR-B1 isoform. *EcR-B1* mutants die prior to pupariation, because they fail to undergo spiracle eversion, shortening, attachment, and cuticular tanning (12). The last type of mutant only effects the production of the EcR-A isoform (20). *EcR-A* mutants have a mid-pupal lethal phase since EcR-A is required for puparium formation and destruction of salivary glands (20). Together, the three EcR isoforms coordinate tissue specific, temporal responses to ecdysone signalling. Specifically, it appears that EcR-B2 is required early in development for moulting, EcR-B1 is required for pupariation and death of larval tissues, and EcR-A is required for events in early metamorphosis involving future adult tissues.

Ashburner's model

The puffing of larval salivary gland polytene chromosomes indicates the presence of genes undergoing active transcription. Based on puffing patterns, Ashburner and his colleagues developed a model to explain ecdysone regulation of metamorphosis (Figure 1.2) (21-23). Ecdysone, bound to its receptor, first triggers the transcription of early response genes, while repressing the expression of late-late genes. The protein product of the early genes usually encodes transcription factors, which cause the expression of a subset of late-late genes, as well as inhibiting the transcription of early genes. A third

class, called the early-late response genes, require the ecdysone/receptor complex, and an early gene product for proper expression.

Ecdysis

Each major developmental transition (hatching, moulting, pupation, and eclosion) requires the shedding of a cuticle, a process known as ecdysis. This is induced by a cascade of neuropeptides that act on ecdysone primed tissues to elicit their response (Figure 1.3). The ecdysis sequence occurs in three stages, pre-ecdysis, ecdysis and post-ecdysis. The first visible cue indicating impending larval ecdysis is pigmentation of the new mouth hooks adjacent to the old ones, which occurs approximately one hour (1-2 moult) or 104 minutes (2-3 moult) prior to ecdysis onset (24). New vertical plates appear 30 minutes prior to eclosion, leading to the double vertical plates (DVP) stage. Within 10 minutes, the old trachea collapse, followed by air-filling of the new trachea, marking the beginning of pre-ecdysis behaviours. The first stage of pre-ecdysis muscle movements is characterized by repetitive anterior to posterior contractions, followed by a second stage of rolling contractions that travel posterior to anterior, ending in head retractions. Ecdysis behaviours begin 25 minutes after DVP. First, one or two forward head thrusts that coincide with shedding of the old tracheal lining detach the old mouthparts. This is followed by backward thrusts to detach the old posterior spiracles, followed by a two to five minute rest period. Ecdysis is via a forward lateral turning escape movement, which results in the shedding of the cuticle.

Pupal ecdysis (commonly referred to as pupation), while not a “classical” moult, involves secretion of a new cuticle within the former larval cuticle that was tanned and hardened to become the pupal case. Pupation occurs approximately 12 hours after pupariation in Drosophila (25). Pupal pre-ecdysis begins with rhythmic retractions of the posterior of the organism away from the puparium (26). Ecdysis occurs within 10 minutes and is characterised by posterior to anterior peristaltic waves that cause head eversion, shedding of the larval tracheae, and rapid extension of the appendages. This is followed by post-ecdysis abdominal contractions, that aide in giving the organism its final adult form within the pupal case.

Adult ecdysis, or eclosion, involves the organism emerging from the pupal case. Pre-ecdysis involves tracheal air-filling approximately one hour prior to eclosion (26).

Three to five minutes before eclosion, the organism extends its ptilinum to rupture the operculum. Peristaltic contractions then propel the organism out of the pupal case. Post-eclosion, the organism swallows the air that is required for the cuticle and wing expansion that occurs within one hour of eclosion. Within three hours, tanning and hardening of the adult cuticle occur (26).

The ecdysis neuropeptide cascade

A decline in ecdysone titre is required to initiate the release of the ecdysis neuropeptide cascade, otherwise, ecdysis is blocked (Figure 1.3) (27). It has been suggested that a drop in steroid hormone titre signifies an organisms readiness to shed its cuticle (28). Most of what is known about ecdysis comes from studies in *Manduca sexta*, with complementary work done in *Bombyx mori* and Drosophila.

Ecdysis-triggering hormone

The first ecdysis neuropeptide to be released when ecdysone titres decline is ecdysis-triggering hormone (ETH) (Figure 1.3). ETH is secreted from seven pairs of epitracheal Inka cells (24). Interestingly, preliminary studies in Drosophila and a Manduca cell line show that ecdysone upregulates *eth* gene expression (29,30). This likely occurs through binding of an ecdysone bound EcR/USP receptor complex to an EcRE located upstream of the gene (31-33). However, additional factors, such as Cryptocephal (CRC), a transcription factor expressed in the Inka cells (33,34), are also required for proper *eth* expression. There are putative CRC binding sites upstream of *eth*, and *crc* mutants severely repress *eth* transcription, resulting in ecdysis defects (33). Furthermore, a putative binding site for the products of the early response gene, *E74*, is located within the 382 bp enhancer that is required for proper *eth* expression. Together, these data suggest that *eth* is an early-late ecdysone response gene.

High ecdysone levels cause accumulation of ETH in Inka cells, and conversely, also appear to suppress the release of ETH into the haemolymph. Injection of ecdysone into Manduca causes a dose-dependent delay in ETH release and ecdysis onset (32). In fact, Inka cells are not competent to release ETH until ecdysone levels have dropped (35).

The Drosophila *eth* gene encodes two peptides, ETH1 and ETH2 (36). In Drosophila larvae, both tracheal inflation and the entire ecdysis sequence are activated by ETH1. On the other hand, ETH2 induces the ecdysis behaviour only after a

corresponding delay in pre-ecdysis (24). Both ETH1 and ETH2 can induce the entire pupal and adult ecdysis sequence, although ETH1 is ten times more potent (24,37). Deletion of the *eth* gene leads to severe behavioural defects that cause lethality at the first larval moult, in part due to a loss of tracheal air-filling (24). These data suggest that ETH1 and ETH2 initiate ecdysis by triggering pre-ecdysis.

The ETH receptor

ETH can initiate pre-ecdysis by binding to its receptor, ETHR. In addition to its role in ETH synthesis, several lines of evidence suggest that ecdysone is necessary for *ethr* expression in the CNS. First, organisms with low ecdysone levels do not respond to ETH injections, while those with high ecdysone titres do (31,32,38). Second, CNS sensitivity to ETH can be restored by injection of ecdysone into freshly ecdysed or feeding larvae that normally contain low ecdysone titres (31). Lastly, neuron-specific expression of *ethr* mRNA correlates with the peak in ecdysone (37,39). The Drosophila and Manduca *ethr* genes encode two receptor subtypes, ETHR-A and ETHR-B (39-41). The Drosophila ETHR isoforms are expressed in mutually exclusive populations of neurons in the CNS, suggesting that they mediate different functions (37). The neurons expressing ETHR-B have not yet been characterised. ETHR-A is restricted to neuropeptide expressing cells, including the lateral abdominal neurons that produce kinin, the ventrolateral FMRFamide neurosecretory cells, the ventromedial eclosion hormone (EH) producing neurons, and the dorsolateral neurons that contain crustacean cardioactive peptide (CCAP), myoinhibitory peptides (MIPs), and bursicon. This suggests that ETH can exert its effects through ETHR-A in these cells. Studies in Manduca have shown that the neuropeptide corazonin is responsible for the initial release of a small amount of ETH (42). ETH, in turn, causes the release of EH, and these neuropeptides act in a positive feedback loop to increase the release of the other (Figure 1.3) (43).

Eclosion hormone

The *eh* gene encodes a single polypeptide (44). Flies that lack EH expressing neurons (EH-KO) can be created by using the bipartite Gal4/UAS system (45), to express the apoptosis gene *reaper* (*rpr*) under the control of an EH-Gal4 driver (46). EH-KO flies fail to release ETH in only 59.1% of Inka cells (47), consistent with a potential role

for corazonin in ETH release. EH-KO flies have a tracheal air filling defect that kills most organisms in the larval stage. Detailed analysis shows that EH-KO larvae also have reduced strength of pre-ecdysis behaviours (47), although there is no difference in the length of pre-ecdysis and ecdysis. Those organisms that survive to adulthood have major defects at the adult ecdysis (46). The first defect observed is in the timing of tracheal air-filling, followed by a lengthened latency from ptilinum extension to emergence in pre-ecdysis. Defects in eclosion behaviours were also observed. The time interval between the onset of ptilinum extension and operculum rupture was 20 times longer than controls, the length of time from operculum rupture to head emergence was five times longer, and the time interval from head emergence to completion of eclosion was three times longer. Finally, EH-KO flies failed to expand their wings and showed a delay in tanning of the cuticle post-eclosion. The EH receptor has not yet been identified; however, it is known that EH acts via the accumulation of cGMP (48,49). It appears that, together, ETH and EH control pre-ecdysis events, and trigger the release of the neuropeptides responsible for ecdysis and post-ecdysis behaviours.

Crustacean cardioactive peptide

EH causes the release of CCAP (Figure 1.3) (48,50). This neuropeptide is proposed to control the ecdysis motor program in Manduca (51) and is released immediately prior to the onset of ecdysis in Drosophila (47). Genetic ablation of CCAP expressing cells (CCAP-KO) causes only minor defects at larval ecdyses. CCAP-KO organisms display a modest lengthening of the pre-ecdysis period (26), and take longer to shed their cuticle due to changes in peristaltic contractions. Thus, CCAP effects only the larval ecdysis behaviours, not pre-ecdysis behaviours.

Most CCAP-KO organisms die at pupation. This is marked by a failure of the head to completely evert, a loss of larval tracheal shedding, and improperly extended appendages (26). A defect in initiation of the ecdysis behavioural sequence, shown by an increased pre-ecdysis period that is followed immediately by a shortened post-ecdysis, is responsible. The small number of CCAP-KO flies that survive to adulthood show minor defects in the eclosion motor program. The time it takes for tracheal air filling is extended. The abdominal contractions that are used to propel the organism forward are induced normally, but the organism fails to expand its body to create the traction that is

needed, making the contractions inefficient at propelling the organism forward. CCAP-KO adult flies also fail to expand their wings and tan post-eclosion.

The CCAP receptor

A G-protein coupled receptor proposed to encode the CCAP receptor has been discovered (52,53). This receptor likely stimulates adenylyl cyclase, causing an increase in the amount of cyclic adenosine mono-phosphate (cAMP), which can then activate protein kinase A (PKA). Expression of a dominant negative form of PKA in CCAP neurons recapitulates the CCAP-KO adult phenotype, supporting this hypothesis (54).

Bursicon and its receptor Rickets

Genetic ablation of CCAP neurons does not just eliminate CCAP expression, but also expression of MIPs and Bursicon (Figure 1.3), as these neuropeptides co-localize with CCAP (26,54,55). It has been proposed that release of MIPs and CCAP into the haemolymph regulates the heart rate and blood pressure needed for cuticle expansion (37). The other neuropeptide that co-localizes with CCAP, Bursicon, is a heterodimeric neuropeptide composed of an α and a β subunit, which are encoded by *bursicon* (*burs*) and *partner of bursicon* (*pburs*) genes, respectively (55-57). Both *burs* and *pburs* transcripts are present in embryos, third instar larvae, and pupae (57). The embryonic and larval roles of Bursicon have not been determined; however, it has long been known as the post-eclosion hormone that was first identified through neck-ligation experiments (58). Neck-ligation of flies at eclosion prevents tanning, while flies neck-ligated 30 minutes after eclosion tan normally (59). Hypomorphic alleles of *burs* show a delay in tanning following eclosion, and a failure in cuticle and wing expansion, but no defects at the larval or pupal ecdyses (56). Bursicon acts through its receptor Rickets (RK) (55,57,60). RK is a G-coupled protein receptor and its activation triggers activity of adenylyl cyclase and a subsequent increase in cAMP (61). Both *burs* and *rk* mutants fail to expand their wings and show a delay in tanning following eclosion (56,60). Interestingly, defects in tanning can be rescued in neck-ligated flies and *rk* mutants by injection of 8-bromo-cyclic adenosine mono-phosphate (8-Br-cAMP), a membrane permeable analog of cAMP (60).

Post-eclosion tanning

Although EH-KO and CCAP-KO organisms have defects at all ecdyses, those that survive until adulthood have the same phenotypes as *burs* and *rk* mutants. These organisms fail to expand their wings and cuticle post-eclosion and show a delay in tanning. This phenotype is mirrored in flies expressing a dominant negative form of EcR under the control of an EH driver (18), demonstrating the importance of ecdysone as a trigger for this cascade. The failure of these flies to undergo post-eclosion expansion and tanning indicates a loss of signalling that controls these processes. Tanning commonly refers to the colouration that accompanies the hardening of the cuticle. In Drosophila, the mature cuticle is light brown over most of the organism due to sclerotization, but the abdominal tergites are black where melanisation occurs. Both processes require metabolites of dopamine (DA) (Figure 1.4) (62).

Tyrosine hydroxylase

Tyrosine hydroxylase (TH), encoded by *pale* (*ple*) catalyzes the conversion of tyrosine to dihydroxyphenylalanine (Dopa), the first and rate limiting step in the production of DA (Figure 1.4) (63-65). In Drosophila, *ple* is alternatively spliced to produce neural and epidermal specific transcripts (Figure 1.5) (66). The epidermal transcript contains the third and fourth exons that are not included in the neural mRNA. Null mutations of *ple* are homozygous lethal, and supplying the epidermal but not the neural form of the enzyme on a transgene, rescues lethality (67). TH activity is regulated at a variety of levels in vertebrates, including transcriptional regulation, alternative splicing, RNA stability, DA feedback inhibition and protein kinase activation (68-71). Both transcriptional regulation and alternative splicing regulate TH activity in Drosophila (66,67). Similar to mammals, the co-factor tetrahydrobiopterin (BH_4) and ferrous iron are essential for Drosophila TH activity (72,73). The additional exons in the epidermal transcript of *ple* encode a very acidic section of 71 amino acids inserted into the regulatory domain of TH that reduces its sensitivity to DA inhibition *in vitro* (72). Furthermore, Drosophila TH is activated, *in vitro*, by phosphorylation of a Serine (Ser) residue that is the 32nd amino acid in the protein (Ser32), a site homologous to vertebrate Ser40 (72), the major site of PKA phosphorylation and activation (68,70). PKA phosphorylation of Ser32 only increases TH activity in the presence, but not the absence,

of DA (72,73). It has been proposed that phosphorylation of Ser32 increases the activity of the epidermal isoform of TH by reducing the ability of the protein to bind inhibitory DA, and by increasing the rate of binding to BH_4 (72). Although phosphorylation of more than one residue alters TH activity in mammals (68), it appears that Ser32 is the only site phosphorylated on Drosophila TH (72). Interestingly, both CCAP and Bursicon signalling activate PKA by elevating levels of cAMP (54,60), suggesting that either of these neuropeptides may be involved in the activation of TH to regulate post-eclosion tanning.

Dopa decarboxylase

Dopa decarboxylase (DDC), encoded by *Ddc* (74), catalyzes the second step in the synthesis of DA, by converting Dopa to DA (Figure 1.4) (75). DDC also catalyzes the conversion of 5-hydroxytryptophan to serotonin. Alternative splicing generates neural and epidermal specific isoforms of *Ddc* (Figure 1.5) (76). The epidermal-specific transcript lacks the neural-specific second exon. *Ddc* mutant homozygotes are lethal and rare escaper flies exhibit defects in cuticular tanning and die within 24 hours of eclosion (77). Similar to *ple* mutants, using transgenes to supply the epidermal isoform of DDC to *Ddc* mutants rescues lethality (76). Furthermore, the neural role of DDC is well established; however, only 6% of DDC activity is in the brain of third instar larvae, demonstrating the importance of epidermal DA production (74). Unlike TH, DDC has never been shown to be regulated at an activational level. DDC activity rises in the late third instar and reaches a peak at pupariation, when large amounts of activity are required for rapid tanning of the pupal case, and then drops (78). DDC activity levels rise before, and peak at eclosion. The early rise in activity is necessary for pigmentation of the pharate adult bristles and epidermis, and levels remain high for tanning of the adult cuticle following eclosion.

The *Ddc* gene is subject to complex transcriptional regulation to achieve the developmental regulation of its activity described above. In fact, *Ddc* is a good example of an early-late ecdysone response gene, since both ecdysone and an early gene product are required for proper expression in the epidermis of third instar larvae (79,80). *Ddc* transcription is induced at this time because DA is required for the rapid tanning of the pupal case that occurs at pupariation. The ecdysone receptor binds to an EcRE that is

located 97 bp upstream of the transcription start site to induce *Ddc* expression (79). The EcR-B1 isoform is primarily responsible for *Ddc* transcriptional induction. In addition, the product of the early response gene, *broad* (*br*), is required for *Ddc* expression in late third instar larvae (80). The *br* gene encodes four isoforms, Z1-Z4 (81). The Z2 isoform of BR acts through three binding sites 700 to 800 bp upstream of the *Ddc* transcription start site to induce transcription (80,82). Together, the ecdysone-receptor complex and BR-Z2 activate the expression of *Ddc* that is necessary for rapid tanning of the pupal case. There are also 17 BR binding sites, representing all four BR isoforms, within the first intron of *Ddc* (83). The sequence within this intron is required for proper expression of *Ddc* in pharate adults (80), corresponding to the time when pigmentation of the bristles and epidermis occurs. DDC activity is high at hatching and both moults (78). The DA produced by DDC is required for the production of melanin and sclerotin.

Metabolism of dopamine

There are two types of melanin, Dopa melanin and DA melanin (Figure 1.4). Dopa melanin production is under the control of the *yellow* gene (84,85), although the exact function of its product is unknown. Two yellow-related proteins, Yellow-f and Yellow-f2, which convert dopachrome to 5,6-hydroxyindole are also important (86). DA is converted to DA melanin through an uncharacterized pathway. There are also two pathways that lead to the production of sclerotin. DA can be converted to N-acetyl DA (NADA) through the action of arylalkylamine N-acyltransferases (aaNAT) (87), resulting in a colourless sclerotin (88). Lastly, DA is converted by N-β-alanyl DA (NBAD) synthase, encoded by *ebony*, to NBAD (84,89). This results in the production of a yellow or light tan coloured cuticle. In addition, NBAD hydrolase, encoded by *tan*, can convert NBAD to DA (88). The product of the *black* (*b*) gene is also necessary for the production of NBAD (90). *b* mutants have been shown to have low aspartate decarboxylase activity (91), and abnormally high β-alanine transaminase activity (92), and reduced NBAD pools (90). All of the melanin and sclerotin precursors are extracellularly polymerized and cross-linked to cuticle proteins, probably through the actions of a common set of enzymes. It has been proposed the phenol oxidase (PO), an enzyme important for the production of melanin in the wound response, may play a role, although its role in the developing cuticle is not understood (93).

In Chapter 2 of this thesis, I demonstrate how some post-eclosion events are controlled by the ecdysis neuropeptide cascade. I use cuticular tanning as a convenient visible marker of the temporally precise post-eclosion developmental progression. Levels of both the *ple* and *Ddc* transcripts begin to accumulate prior to eclosion, coincident with the onset of pigmentation of the pharate adult bristles and epidermis. Since DDC activity is high prior to the post-eclosion onset of tanning, a different factor must be regulated in order to switch on tanning. Transcriptional control of *ple* does not regulate the onset of tanning, since *ple* transcript levels remain unchanged from 24 hours before to 12 hours after eclosion. TH protein present prior to eclosion is degraded and no TH activity can be detected at eclosion. However, TH protein rapidly accumulates within an hour following eclosion and I provide evidence that CCAP controls this process. Furthermore, I show that TH is transiently activated during tanning by phosphorylation at Ser32, as a result of Bursicon/RK signalling. I conclude that the ecdysis hormone cascade acts as a regulatory switch to control the precise onset of tanning by both translational and activational control of TH.

Part 2: The Drosophila innate immune response

The ability to combat infection is necessary for survival of multicellular organisms. In mammals, invading pathogens are destroyed through the combined action of the immediately induced innate immune response, and the slower, more specific, acquired immunity. The complex adaptive immune response has only evolved recently, and the majority of organisms combat infection using only an innate immune response. Innate immunity has been best studied in Drosophila, where immunity can be divided into humoral and cellular branches. This organism serves as an exceptional model to study medically relevant aspects of innate immunity because of the conservation of the core signalling pathways from insects to humans. Three mechanisms contribute to the Drosophila innate immune response: 1. transient synthesis of antimicrobial peptides (AMPs) at both the wound site and in the fat body followed by secretion into the haemolymph in a systemic response; 2. blood clotting, melanin formation, and opsonisation following septic injury; and 3. phagocytosis or encapsulation of invading organisms by specialized haemocytes (94).

Antimicrobial peptides

There are approximately 20 immune-inducible AMPs encoded in the Drosophila genome (95). These AMPs can be grouped into seven classes, according to sequence similarities (Table 1.1). The AMPs, with the exception of the 25 kD Attacin, are all less than 10 kD, and have a broad range of antibacterial and/or antifungal activity. Gram negative bacteria are combated by Diptericin, Drosocin, and Attacin (96-98). Defensin is important for killing gram positive bacteria (99). Drosomycin and Metchnikowin are induced following fungal infection (100,101). Finally, Cecropins have both antibacterial and antifungal properties (102,103). Gram negative, gram positive and fungal infections induce different patterns of AMP expression (104). Fat body synthesis and secretion of AMPs into the haemolymph are the hallmarks of the humoral immune response. Interestingly, the barrier epithelia, which consists of the hypoderm below the cuticle and the epithelia of the gut and tracheae, are responsible for localized production of AMPs at the site of septic injury (105,106). Furthermore, the barrier epithelia constitutively express low levels of AMPs in the absence of infection, thereby creating a microbicidal surface that prevents infection (107).

Pathogen recognition

The differential induction of AMP gene transcription is achieved through specific activation of the Toll pathway by gram positive bacteria or fungi or the Immune deficiency (IMD) pathway by gram negative bacteria. Pattern recognition receptors (PRRs) detect infecting microbes by direct contact with microbial molecules. In Drosophila, peptidoglycan recognition proteins (PGRPs) are the PRRs that recognize specific forms of peptidoglycan (PGN) (108), a component of the bacterial cell wall. Insect PGRPs are very similar to those identified in other organisms (109-111). All PGRPs share a 160 amino acid domain (PGRP domain) with similarities to bacteriophage T7 lysozyme, a zinc-dependent *N*-acetylmuramoyl-L-alanine amidase. There are two subgroups of PGRPs in Drosophila. The first consists of the catalytic PGRPs (PGRP-SC, -LB, and -SB) that have zinc-dependent amidase activity (112-114). The second group contains PGRP-SA, -SD, -LA, -LC, -LD, -LE, and –LF. These PGRPs lack amidase activity, but can bind to and recognize PGN, thereby functioning as PRRs.

Unlike vertebrates, lipopolysaccharide (LPS), the major component of the gram negative cell envelope, does not activate the Toll or IMD pathway (115,116). PGN contamination of commercial LPS preparations explains the initial reports that LPS stimulated the Drosophila immune response. In general, gram positive bacteria have a lysine (Lys) residue at the third position in the PGN chain, while gram negative PGN has meso-diaminopimelic acid (DAP). DAP-type PGN binding to membrane-bound PGRPs activates the IMD pathway, while the Toll pathway is activated by secreted PGRPs that sense Lys-type PGN (115).

PGRP-LC is the major sensor of DAP-type PGN that activates the IMD pathway (Figure 1.6) (117). In addition, PGRP-LE expressed extracellularly enhances the PGRP-LC-mediated recognition (118,119), while when it is expressed cytoplasmically, it can bypass the need for PGRP-LC signalling altogether (120). Several amidase PGRPs, including PGRP-LB and PGRP-SC, have an important role in regulating IMD pathway activity by degrading PGN (114,121). Their amidase activity removes peptides from PGN, converting it into non-immunostimulatory fragments, thereby attenuating the immune response. In fact, in the haemolymph, PGRP-LB establishes a negative feedback loop that ensures that the immune response to gram negative bacteria is elicited appropriately (114). This prevents excessive demands on host resources and avoids the potentially dangerous consequence of over-expression of AMPs.

In the haemolymph, the Lys-type PGN of gram positive bacteria is recognized by PGRP-SA (122) complexed with the gram negative binding protein GNBP1 (Figure 1.6) (123,124). It has been proposed that GNBP1 hydrolyzes Lys-type PGN into small fragments that can then be detected by PGRP-SA (125,126). In addition, PGRP-SD mediates Toll activation by a subset of gram positive bacteria, with partial redundancy to PGRP-SA/GNBP1 (127). The GNBP, GNBP3, has homology to a lepidopteran fungal binding protein (128), and appears to be the PRR responsible for sensing cell wall components of fungi, including glucans, resulting in activation of the Toll pathway in Drosophila (Figure 1.6) (129).

The Toll pathway

In contrast to vertebrate Toll receptors, which sense microbes directly, the Drosophila Toll pathway is activated by binding of a cleaved form of Spätzle to the Toll

receptor (Figure 1.6) (130-132). PRR activation of several serine proteases (SPs), that undergo zymogen activation, leads to the eventual cleavage of Spätzle (133). The SP cascade required to activate Spätzle in immunity is different from the one mediating the extensive roles that the Toll pathway has in embryonic development (132,134). Five SPs have been identified that are required for Toll activation following gram positive infection (135). The terminal protease, Spätzle processing enzyme (SPE), which catalyzes the cleavage of Spätzle, is very similar to the embryonic Spätzle processing enzyme, Easter (135,136). Inappropriate activation of SP cascades is prevented by SP inhibitors called Serpins (137).

Four of the five SPs that are necessary for gram positive Toll induction, including SPE, are also required for fungal Toll induction (135,136). The fungal-specific activation of Toll is mediated by the SP Persephone (PSH) (138) and the inhibitory serpin, Necrotic (Figure 1.6) (139). Interestingly, a secreted fungal protease, PR1A, directly processes PSH into its catalytic form, thereby activating the Toll pathway and causing AMP expression (129). Therefore, it appears that fungal activation of Toll occurs through two pathways: fungal protease cleavage of PSH; and GNBP3 recognition of fungal glucans that triggers an SP cascade. Both processes result in the activation of Toll through SPE mediated Spätzle cleavage.

At the plasma membrane, the Toll receptor dimerizes following binding of a processed Spätzle dimer (Figure 1.6) (130,131). Activation of Toll causes recruitment of the death-domain (DD) containing adaptor protein myeloid-differentiation factor 88 (MyD88), which interacts with the Toll receptor through their respective Toll/IL-1 receptor (TIR) domains (140). The DD is required to transmit the signal to the DD containing adaptor protein Tube, which then activates Pelle kinase, the homolog of the IL-1 receptor-associated kinase (134,141). Subsequently, through an unknown mechanism, the inhibitory IκB homolog, Cactus, is phosphorylated and degraded by the proteasome (142). This releases the NFκB transcription factors Dorsal (DL) or Dorsal-related immunity factor (DIF) that can then translocate to the nucleus to effect transcription of their target genes (143,144).

The specific Toll responsible for immune induction of gene expression has not yet been identified. In fact, there are eight Drosophila Toll proteins, many of which have

been implicated in immune signalling. Toll signalling upregulates many of the members of the Toll pathway (132,145), including Cactus, which establishes a negative feedback loop (142). Except for mutations in *dl* or *cactus*, flies homozygous for hypomorphic mutations in Toll pathway components, that survive past embryogenesis, lose immune gene expression, and are susceptible to fungal and gram positive infection (132,146). DIF and DL appear to be redundant in larvae, while DL is dispensable in the adult immune response (147-149). Since Cactus is a repressor of DIF and DL, mutation in *cactus* would lead to precocious translocation of DIF and DL to the nucleus, which would constitutively induce immune gene expression.

The IMD pathway

The Drosophila *imd* gene encodes a DD-containing protein (Figure 1.6) with homology to the Receptor Interacting Protein 1 of the Tumour Necrosis Factor (TNF) receptor pathway (150). It was originally identified because a mutation in *imd* caused reduction of expression of several AMP genes (151-153). Once PGRP-LC binds to PGN, PGRP-LC transduces the signal to IMD (117,120,154,155). IMD interacts with the adaptor protein called Fas-Associated Protein with Death Domain (dFADD) (156,157). dFADD associates with the caspase Dredd through their respective death effector domains (DED) (158,159). In addition, transforming growth factor (TGF) β-activated kinase 1 (TAK1) is activated by IMD signalling (160,161). The inhibitor of apoptosis protein, DIAP2 (162-164) is a putative E3 ubiquitin ligase that has a baculovirus IAP repeat (BIR) domain and a really interesting new gene (RING) domain, that may activate TAK1. TAK1 and its adaptor, TAK1 binding protein (TAB2) (162,163,165) are thought to activate the IKK signalling complex in an IMD and possibly a dFADD-dependent manner (156) although the exact link between IMD and the IKK complex is unknown. The IKK signalling complex is composed of IKKβ/ird5 and IKKγ/kenny (166-168). The IKK complex can phosphorylate the NFκB transcription factor Relish (REL) (168,169), thereby targeting it for cleavage by an unknown mechanism. Dredd interacts with phosphorylated REL, and is required for cleavage (170,171); however, it has never been shown to be directly responsible for cleavage. Once cleaved, REL translocates to the nucleus to elicit its gene specific transcriptional effects.

Flies bearing mutations in any of the IMD pathway components, such as IMD, dFADD, and TAK1 (Figure 1.6), are viable in the absence of infection, but succumb to gram negative infection (156,157,161). In addition to the core IMD pathway members, several modulators of the IMD pathway have also been identified (162,172-178). One such modulator, defence repressor 1 (DNR1) was shown to repress Dredd-specific immunity gene induction (174). Interestingly, DNR1 also appears to be an inhibitor of the initiator caspases that trigger apoptosis (179). In addition, over-expression of DIAP2 inhibited apoptosis, while deletion of DIAP2 increased caspase activity (164,180). These observations suggest that there is cross-talk between the immune and apoptotic responses.

The JNK pathway

The IMD pathway also induces the activity of the Jun kinase (JNK) pathway due to a bifurcation at TAK1 (Figure 1.6) (181). The Drosophila JNK pathway consists of the MAPK kinase (MAPKK), Hemipterous (HEP), and the JNK, Basket (BSK) (182). All JNKs are activated by dual phosphorylation of a characteristic TPY motif (183). Immune activation of BSK occurs through TAK1 (181), and requires both Dredd and dFADD (175). BSK activates, by phosphorylation, the heterodimeric transcription factor AP-1 (184,185), which is composed of one Fos and one Jun subunit (186,187). The role of JNK in AMP expression is controversial, since a role in both induction (188,189) and repression (190) of AMP expression has been proposed. However, a subset of IMD-independent, JNK-dependent non-AMP immune response genes are induced following gram negative infection (160). JNK signalling activates transient expression of genes prior to the sustained REL-dependent gene expression (191). Proteasomal degradation, through an ubiquitin ligase RING domain protein called Plenty of SH3s (POSH), is responsible for rapid termination of JNK signalling (177).

The JNK pathway is required for embryonic dorsal closure (182,185,192) and apoptosis (193,194). Furthermore, JNK signalling is necessary for wound healing in the adult epidermis (195,196) and the larval wing disc (197). JNK has a role in the cellular immune induction of haemocytes during parasitic wasp infestation (198) and crystal cell rupture following wounding (199). It is therefore not surprising that JNK signalling activates many proteins involved in cytoskeleton remodelling (181). The TNF superfamily ligand, Eiger, is responsible for HEP activation through a TAK1-

independent, small Rho-GTPase-dependent mechanism during crystal cell rupture for wound healing (199). During development, HEP is activated by the actions of the MAPKKK Slipper (200,201). However, it appears that JNK pathway induced apoptosis, which is triggered by Eiger, requires both TAK1 and TAB2, similar to immune activation of JNK (202). There is also a link between the JNK pathway and the Toll pathway, since mutation of Toll pathway components caused a loss of expression of the JNK responsive gene *flightin* (181).

The ERK and p38 MAPK pathways

In addition to the JNK pathway, two other conserved MAPK pathways have been implicated in wound healing and immunity. The first, the extracellular signal-regulated kinase (ERK) pathway, and has been implicated in the Drosophila embryonic wound response (203). It also has extensive roles in Drosophila development, including tracheal branching, cell proliferation, mesodermal patterning, R7 photoreceptor cell differentiation, and differentiation of terminal embryonic structures (204-207). In mammals, ERKs mediate cell proliferation induced by a variety of growth factors and are often downstream effectors of Ras signalling (208). The Drosophila ERK, encoded by the gene *rolled* (*rl*) (207), is activated by its MAPKK, MEK, to transduce the ERK signal (209,210). ERK activation occurs through dual phosphorylation of a conserved TEY motif (183).

The second MAPK pathway is the p38 MAPK family, which has two characterized members (p38a and p38b) (211) that are involved in Drosophila development and the environmental stress response. In vertebrates, p38 MAPKs are activated strongly by cellular stresses and inflammatory cytokines (208). Drosophila p38a MAPK signalling is important for the stress response to heat shock, dry starvation, and oxidative stress, but not osmotic shock (212). On the other hand, p38b plays a role in TGFβ signalling during wing morphogenesis (213), and the response to osmotic stress (214). In addition, transcripts of both genes are maternally deposited in the embryo and are expressed throughout embryonic development (211). It has been proposed that both p38a and p38b play a role in the attenuation of the immune response. Consistent with this proposal, the *p38a* gene was shown to be immune inducible by microarray analysis (181). Interestingly, p38 MAPKs, along with JNK and ERK, appear to have a role in *E.*

coli induced phagocytosis in *Ceratitis capitata* (215). p38 MAPKs are activated by dual phosphorylation by a MAPKK at a characteristic TGY site (183). The homolog of human MKK3, encoded by *licorne* (*lic*) (216) is a MAPKK that can activate p38 (217). Lic is involved in the regulation of the localization of cell fate determinants during oogenesis, thereby playing an essential role in anterior-posterior and dorsal-ventral patterning (216). Furthermore, the MAPKKK, MEKK1 is responsible for regulation of p38 signalling (217,218).

The cellular immune response

The Drosophila blood cells, called haemocytes, elicit the cellular innate immune response. There are three types of blood cells in Drosophila: plasmatocytes, crystal cells, and lamellocytes (219-223). Plasmatocytes constitute the major component of the haemolymph. During development, the primary role of the plasmatocytes is phagocytosis, particularly during embryogenesis and metamorphosis, for clearing of the cells that were fated to die (221,224-227). The plasmatocytes are also important in the immune response for phagocytosis of invading bacteria and fungi (228,229), secreting AMPs (230), and possibly signalling to distant tissues about invading microbes (231,232).

Crystal cells constitute 5% of the haemocyte population in embryos and larvae, but disappear at the onset of metamorphosis (219,233). The zymogen prophenol oxidase (PPO) that is stored within these cells creates characteristic crystalline inclusions (234). Following wounding, PPO is activated by a SP proteolytic cascade to produce active PO. PO catalyzes several reactions in the synthesis of melanin to produce a plug at the wound site (Figure 1.7). Therefore, crystal cells are very important to formation of a melanised wound plug.

The third type of haemocyte, the lamellocyte, is rarely observed in the absence of parasitic wasp infestation (220,221,235,236). Parasitic wasps lay eggs in young Drosophila larvae and in defence, the lamellocytes form a multilayered capsule around the egg (237). Melanisation of the capsule then kills the egg. Cytotoxic intermediates of melanin synthesis likely add to the killing effect (238). PO appears to have a role in the melanisation of the lamellocytes within the capsule (Figure 1.7) (237). However, the

melanisation response is severely compromised in *Ddc*^{ts2} flies, suggesting that DDC activity is necessary in the encapsulation response (239).

Wound healing

The primary layer of defence against infection involves the barrier epithelia of the epidermis. Natural infection with a variety of microorganisms can occur through the tissues of the trachea and gut. In general, septic wounding is required to cause a systemic infection. While formation of a melanin clot is not specifically an immune response, it is essential at the wound site to prevent haemolymph loss following septic injury. Clots form without the action of PO (240,241); however, PO is required for clot hardening (241,242). The melanin produced at the clot site is a direct consequence of the catalytic activation of the PPO (241) that is stored in the crystal cells (243,244), to produce active PO. In adults, crystal cells do not circulate. At this time, PPO stored in the epidermal cells is activated at the site of injury.

Crystal cell rupture at the wound site is a consequence of JNK signalling triggered by Eiger (199). However, PO activity is also required in the immune response to help destroy invading pathogens (245). Under normal conditions, the melanisation cascade is inhibited by the serpin Spn27A (138,145). Two SPs that may activate PPO, called melanisation protease (MP) 1 and MP2, have been identified (245). MP2 is activated by fungal infection. MP2 and a putative bacterial-induced SP appear to act through activation of MP1. MP1 can then trigger cleavage of PPO. Transcriptional induction of genes in the fat body is important for Spn27A degradation and production of several potential PPO-activating proteases (138,145,246-248). The method of activation of the SP cascade is unknown; however, precocious induction of PGRP-LE causes melanisation in the absence of infection (118), suggesting that it may be the microbial sensor required. In addition, both the Toll and IMD pathways have been implicated in triggering PPO-activation (119,138,145). Since the JNK pathway is required for crystal cell rupture in the absence of infection (199), it may also be involved in infection induced PPO activation.

PO can convert tyrosine to Dopa and Dopa to Dopaquinone (Figure 1.7) (249). In the presence of DDC, Dopa can also be converted to DA, which PO can convert to DAquinone. The quinones can then react non-enzymatically to produce the two types of

melanin, pheomelanin and eumelanin. There is some evidence that other enzymes may participate in melanin synthesis. An additional benefit of melanin production is that the melanised clot traps bacteria, and promotes killing (250). Another potential role for melanin production at the wound site involves the formation of reactive oxygen species and byproducts of the melanin production that have cytotoxic antimicrobial properties (251).

In addition to the role of PO in wound healing, transcription of both *ple* and *Ddc* is induced in the embryonic wound response (203). In this case, transcripts are detectible in the epidermal tissues surrounding the wound site. The transcription factor Grainy head is reported to be responsible for *Ddc* induction; however, deletion of 3 AP-1 sites and a CREB (an ERK inducible transcription factor) binding site upstream of *Ddc* also eliminates expression during the wound response, indicating a potential role for the JNK or ERK pathways in this induction. Consistent with the role of a MAPK in *Ddc* transcriptional induction, injection of the MAPK inhibitor PD98059 into embryos reduced the expression of a *Ddc*-GFP reporter after aseptic injury.

Melanin production in the innate immune response to bacterial infection

In addition to the role of *Ddc* in the embryonic wound response, *Ddc* transcription was induced in larvae of *Tenebrio molitor* infected with *E. coli* (252), and led to increased DDC protein and activity levels. Furthermore, NBAD synthase activity is upregulated in the epidermis of *T. molitor* and *C. capitata* following septic injury with *E. coli* (253). NBAD is important for sclerotin production, and has potent antimicrobial effects when included in the medium used for bacterial cultures. Addition of antioxidants eliminates the killing effect, suggesting that NBAD exerts its effects through spontaneous conversion to reactive quinones. A potential role for sclerotin and melanin production enzymes in the Drosophila innate immune response was shown in microarrays that identified genes induced after bacterial infection (181,246,247). Among the genes induced are *Ddc* (11-fold), *ple* (2-fold), *punch* (5-fold), *Dhpr* (3-fold), *yellow-f* (6-fold), and *black* (2-fold). *Dhpr* and *punch* encode Dihydropteridine reductase and GTP cyclohydrolase I, respectively. These enzymes are responsible for synthesis of the essential TH cofactor BH_4. Interestingly, *punch* transcriptional induction is a JNK-specific, IKK independent immune response (160). None of the other melanin and

sclerotin production genes were shown to be JNK-specific response genes, and their mode of induction is still to be elucidated. The Toll and IMD pathways are good candidates for activation of these genes. However, studies have shown that Toll and IMD together are responsible for only 80% of immune gene induction, suggesting that other pathways must be involved (145).

In Chapter 3 of this thesis, I investigated the role of melanin and sclerotin production in the Drosophila innate immune response to bacteria infection, by analysing the induction of *Ddc* transcription following infection. I found that transcription of the *Ddc* is induced in response to gram negative and gram positive septic injury, but not aseptic wounding. Transcription is induced throughout the epidermis and not specifically at the site of infection. *Ddc* transcripts are detectible within 2 hours, and levels remain high for 6 hours following gram negative infection, and 36 hours after infection with gram positive bacteria. Using *Ddc*-GFP reporter gene constructs, I showed that a conserved AP-1 binding site upstream of the *Ddc* transcription start site is required for induction. However, neither the Toll, IMD, nor the JNK pathways are involved. Rather, *Ddc* transcription depends on a previously uncharacterised member of the p38 mitogen activated protein kinase family, p38c. I propose that *Ddc* induction increases the amount of dopamine, which is metabolized to produce reactive quinones that exert a killing effect on invading bacteria.

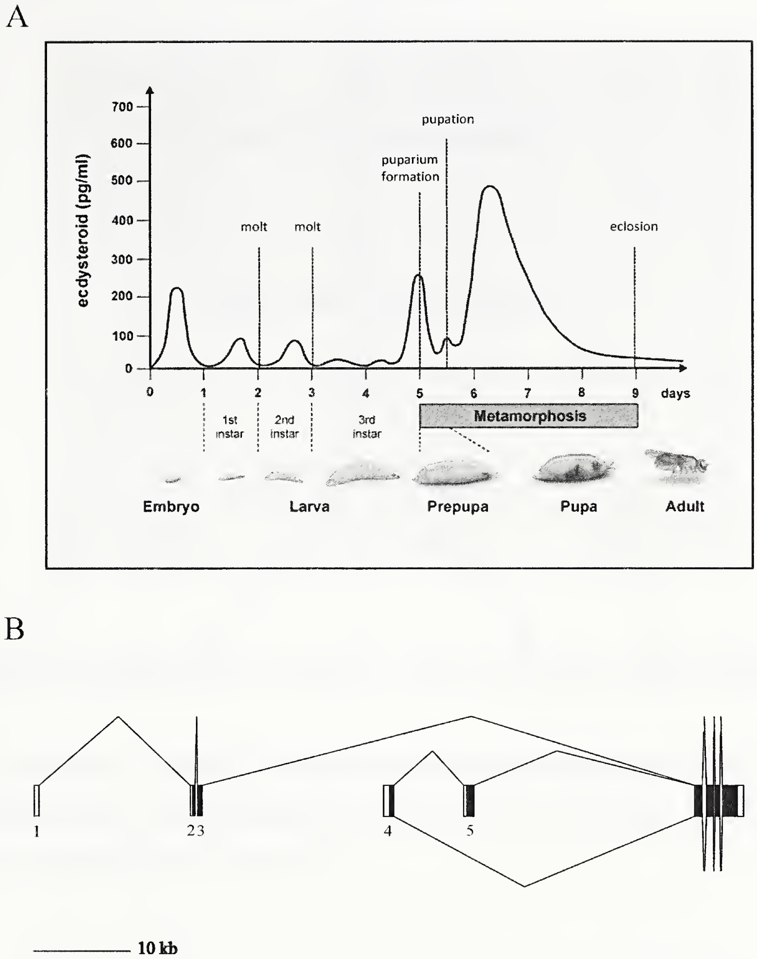

Figure 1.1: Ecdysone titres during the Drosophila life cycle and the ecdysone receptor subtypes. A. Ecdysone titres (pg/mL) from whole body homogenates of Drosophila. The developmental stages during the Drosophila life cycle are shown on a timeline expressed in days. This figure is adapted from (1). B. There are three isoforms encoded in the 78 kb *EcR* gene: *EcR-A* (top alternative splice variant, beginning at exon 1), *EcR-B1* (top splice variant, beginning at exon 4), and *EcR-B2* (splice variant shown below the exon boxes). The coding sequence is shown in black. The coding sequence for *EcR-A* begins in codon 2, for *EcR-B1* in exon 5, and for *EcR-B2* in exon 4. The ligand and DNA binding domains are encoded in the terminal 4 exons that are shared by all three isoforms. This figure is modified from (12) and (11).

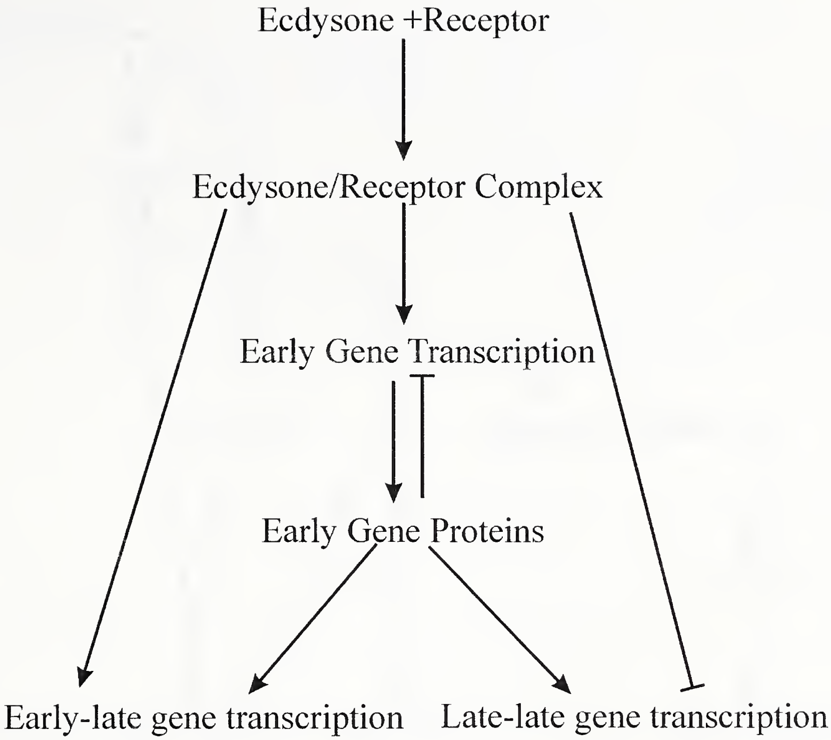
Ecdysone +Receptor
Ecdysone/Receptor Complex
Early Gene Transcription
Early Gene Proteins
Early-late gene transcription
Late-late gene transcription

Figure 1.2: Ashburner's model for ecdysone-regulated gene expression during metamorphosis, based on puffing patterns of larval salivary gland polytene chromosomes. This figure is adapted from (22) and (23).

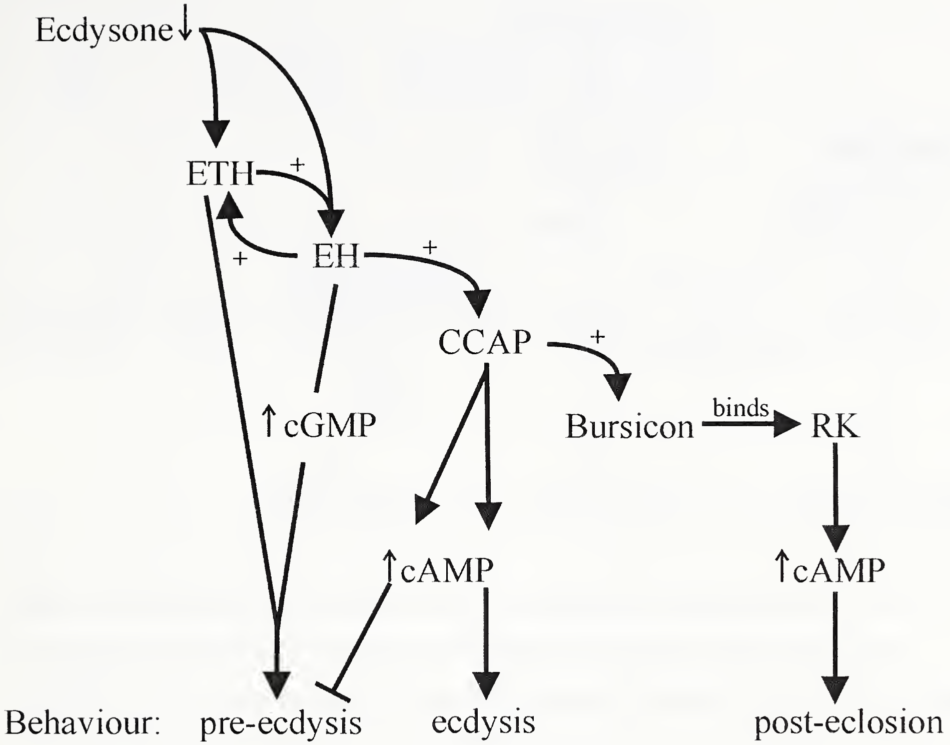

Figure 1.3: The ecdysis neuropeptide signalling cascade. Following a decrease in ecdysone titres, ETH is released, triggering the release of EH. ETH and EH act in a positive feedback loop to increase the release of each other and trigger pre-ecdysis behaviours. EH acts through the second messenger cGMP, and causes the release of CCAP, which shuts off pre-ecdysis and turns on the ecdysis motor program through an increase in cAMP. At eclosion, CCAP also triggers the release of Bursicon, which binds its receptor RK, causing an increase in cAMP that initiates post-eclosion behaviours. This figure is modified from (47) and (46).

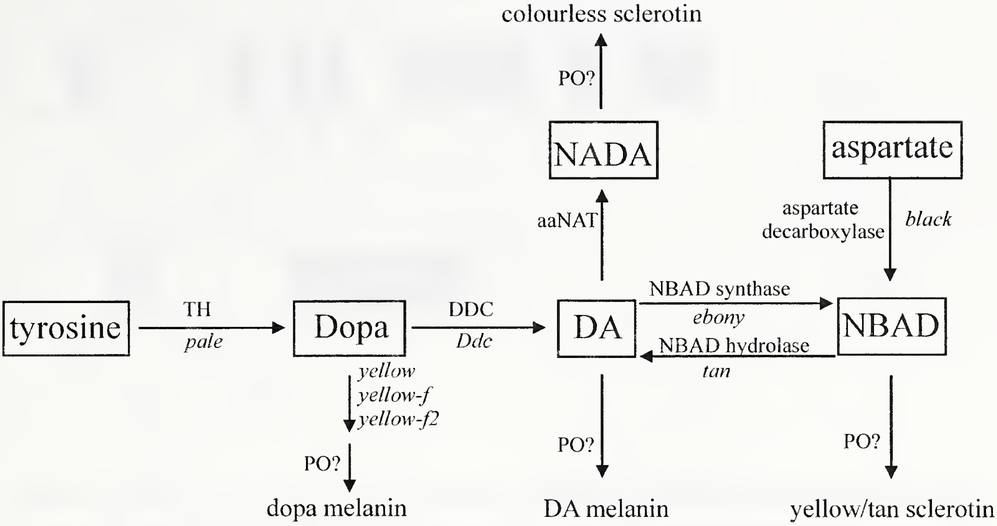

Figure 1.4: Important steps in the production of epidermal melanin and sclerotin. The first and rate limiting step in melanin and sclerotin production is the conversion of tyrosine to Dopa by tyrosine hydroxylase (TH), encoded by *pale*. Dopa can be converted to Dopa melanin by the products of the *yellow*, *yellow-f*, and *yellow-f2* genes. Dopa decarboxylase (DDC) catalyzes the conversion of Dopa to dopamine (DA). An unknown mechanism converts DA to DA melanin. DA is also converted to N-β-alanyl DA (NBAD) by NBAD synthase (encoded by *ebony*) which produces a yellow or tan coloured sclerotin. NBAD is also supplied by the action of aspartate decarboxylase (encoded by *black*) and converted to DA by NBAD hydrolase (encoded by *tan*). Finally, DA can be converted to N-acetyl-DA (NADA) by arylalkylamine N-acyltransferases (aaNAT) to produce a colourless sclerotin. A role for epidermal phenol oxidase (PO) has been proposed for production of both melanin and sclerotin.

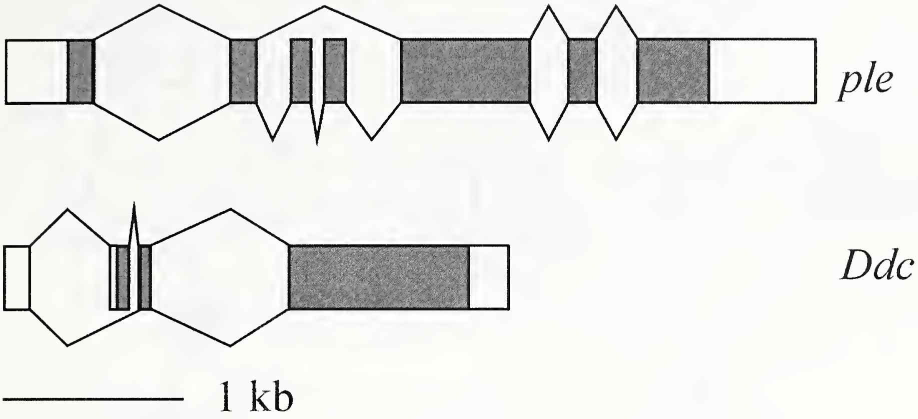

Figure 1.5: Both *ple* and *Ddc* are alternatively spliced to produce neural-specific (shown above exon boxes) and epidermal-specific (shown below exon boxes) transcripts. The coding sequence is shaded in grey.

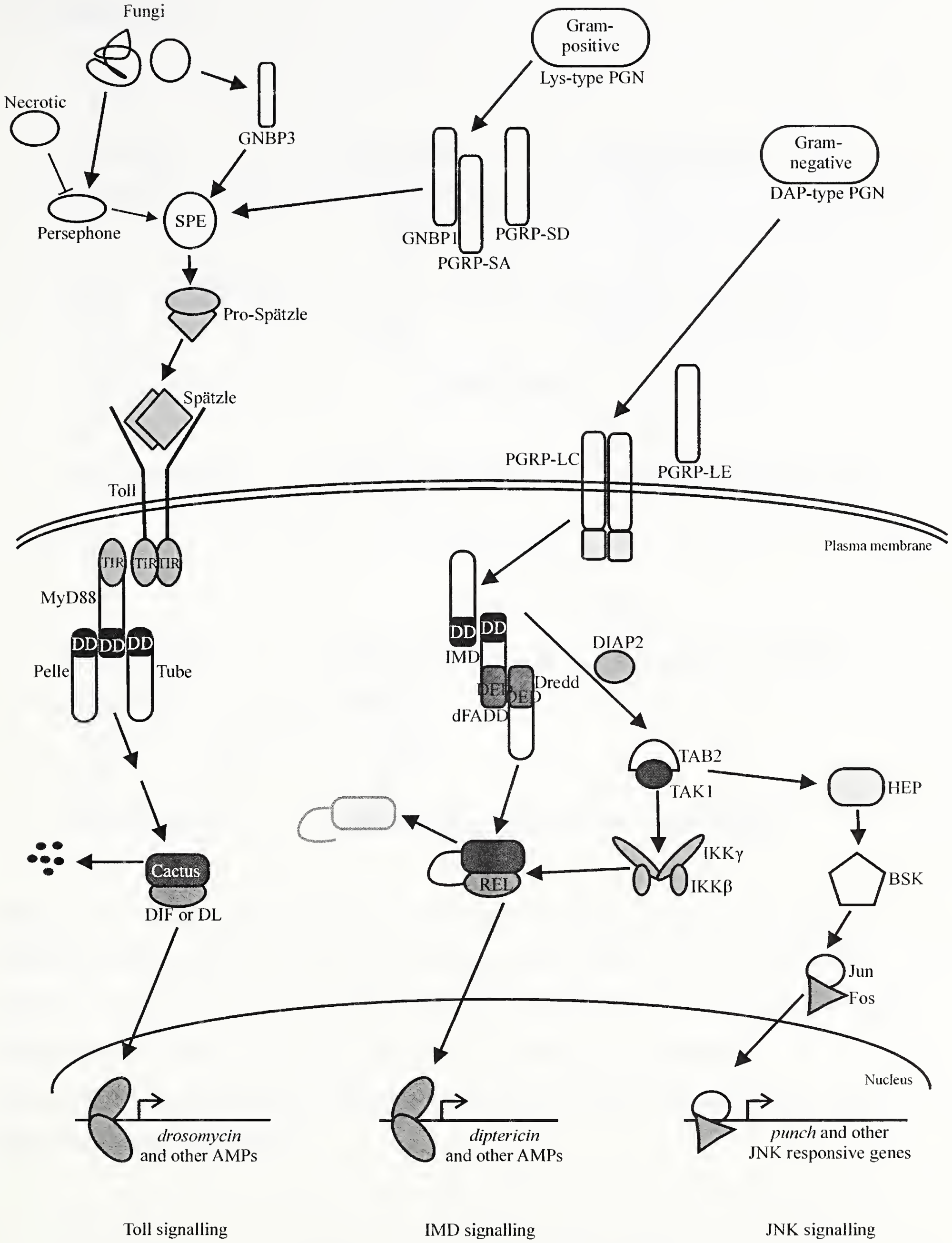

Figure 1.6: Immune induction pathways in Drosophila. The Toll, IMD, and JNK pathways are required for bacterial induction of gene expression (see text for details). This figure is adapted from (94).

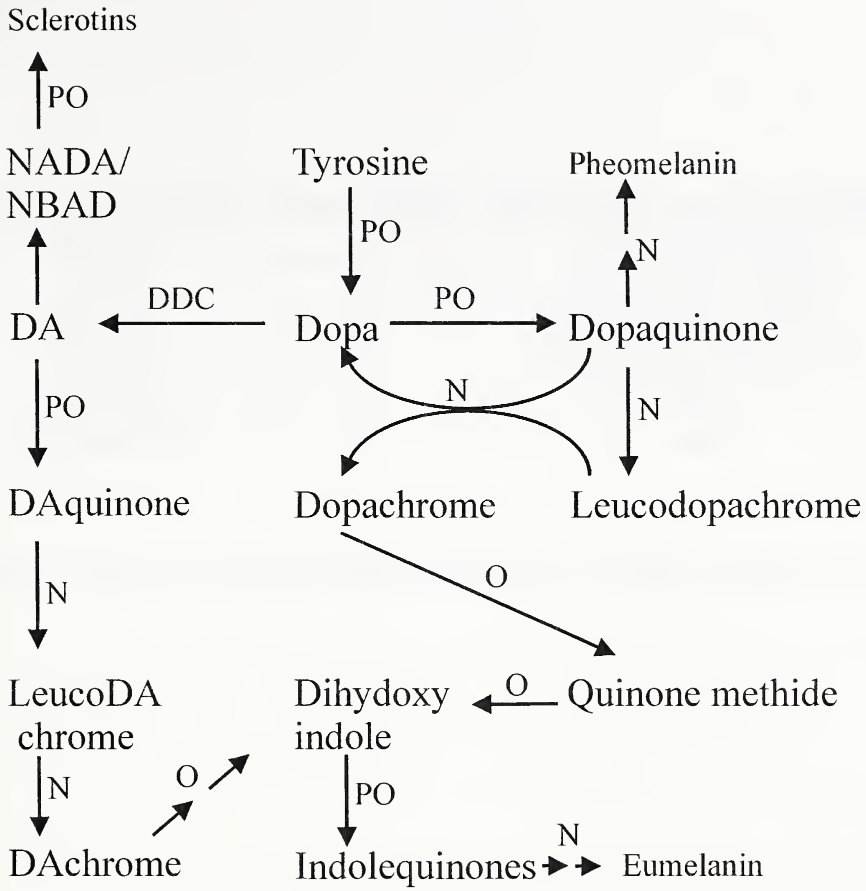

Figure 1.7: Wound/immune induced melanin and sclerotin synthesis in Drosophila requires phenol oxidase (PO) and Dopa decarboxylase (DDC). The products of the reactions, catalyzed by these enzymes, react non-enzymatically (N) to produce melanin. Other enzymes (O) may have a role in melanin synthesis. DA:dopamine; Dopa:dihydroxyphenylalanine; NADA: N-acetyl DA; NBAD: N-β-alanyl DA. This figure is adapted from (249).

Antimicrobial peptide	**Number of genes**	**Main antimicrobial function against**
Diptericin	2	Gram-negative bacteria
Attacin	4	Gram-negative bacteria
Drosocin	1	Gram-negative bacteria
Cecropin	4	Gram-negative bacteria, Fungi
Defensin	1	Gram-positive bacteria
Drosomycin	7	Fungi
Metchnikowin	1	Fungi

Table 1.1: The seven classes of antimicrobial peptides. This table is adapted from (94).

References

1. Riddiford, L. (1993). Hormones and Drosophila development. In: Bate, M. and Martinez-Ariaz, A. (eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 899-940.
2. Berreur, P., Porcheron, P., Moriniere, M., Berreur-Bonnenfant, J., et al. (1984). Ecdysteroids during the third larval instar in *1(3)ecd-1ts*, a temperature-sensitive mutant of *Drosophila melanogaster*. Gen Comp Endocrinol. 54: 76-84.
3. Henrich, V.C., Livingston, L. and Gilbert, L.I. (1993). Developmental requirements for the *ecdysoneless* (*ecd*) locus in Drosophila melanogaster. Dev Genet. 14: 369-377.
4. Mangelsdorf, D., Thummel, C., Beato, M., Herrlich, P., et al. (1995). The nuclear receptor superfamily: The second decade. Cell. 83: 835-839.
5. Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., et al. (1991). The Drosophila *EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell. 67: 59-77.
6. Thomas, H.E., Stunnenberg, H.G. and Stewart, A.F. (1993). Heterodimerization of the Drosophila ecdysone receptor with retinoid X receptor and ultraspiracle. Nature. 362: 471-475.
7. Yao, T.P., Forman, B.M., Jiang, Z., Cherbas, L., et al. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. Nature. 366: 476-479.
8. Yao, T.P., Segraves, W.A., Oro, A.E., McKeown, M., et al. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell. 71: 63-72.
9. Oro, A.E., McKeown, M. and Evans, R.M. (1990). Relationship between the product of the Drosophila *ultraspiracle* locus and the vertebrate retinoid X receptor. Nature. 347: 298-301.
10. Perrimon, N., Engstrom, L. and Mahowald, A.P. (1985). Developmental genetics of the 2C-D region of the Drosophila X chromosome. Genetics. 111: 23-41.
11. Talbot, W.S., Swyryd, E.A. and Hogness, D.S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell. 73: 1323-1337.

12. Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., et al. (1997). Drosophila *ecdysone receptor* mutations reveal functional differences among receptor isoforms. Cell. 91: 777-788.

13. Kim, S.J., Park, J.G. and Lee, C.C. (1999). Transcript titers of ecdysteroid receptor components vary between tissues and stages during Drosophila development. Mol Cells. 9: 61-66.

14. Sung, C. and Robinow, S. (2000). Characterization of the regulatory elements controlling neuronal expression of the A-isoform of the *ecdysone receptor* gene of *Drosophila melanogaster*. Mech Dev. 91: 237-248.

15. Robinow, S., Talbot, W.S., Hogness, D.S. and Truman, J.W. (1993). Programmed cell death in the Drosophila CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. Development. 119: 1251-1259.

16. Truman, J.W., Talbot, W.S., Fahrbach, S.E. and Hogness, D.S. (1994). Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. Development. 120: 219-234.

17. Dai, J.D. and Gilbert, L.I. (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: A cytophysiological analysis of the ring gland. Dev Biol. 144: 309-326.

18. Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., et al. (2003). EcR isoforms in Drosophila: Testing tissue-specific requirements by targeted blockade and rescue. Development. 130: 271-284.

19. Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W., et al. (1998). Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. Development. 125: 2053-2062.

20. Davis, M.B., Carney, G.E., Robertson, A.E. and Bender, M. (2005). Phenotypic analysis of *EcR-A* mutants suggests that EcR isoforms have unique functions during Drosophila development. Dev Biol. 282: 385-396.

21. Ashburner, M. and Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. III. Consequences of ecdysone withdrawal. Dev Biol. 54: 241-255.
22. Ashburner, M., Chihara, C., Meltzer, P. and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. Cold Spring Harb Symp Quant Biol. 38: 655-662.
23. Thummel, C.S. (2002). Ecdysone-regulated puff genes 2000. Insect Biochem Mol Biol. 32: 113-120.
24. Park, Y., Filippov, V., Gill, S.S. and Adams, M.E. (2002). Deletion of the *ecdysis-triggering hormone* gene leads to lethal ecdysis deficiency. Development. 129: 493-503.
25. Bainbridge, S.P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. J Embryol Exp Morphol. 66: 57-80.
26. Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., et al. (2003). Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior. Development. 130: 2645-2656.
27. Curtis, A.T., Hori, M., Green, J.M., Wolfgang, W.J., et al. (1984). Ecdysteroid regulation of the onset of cuticular melanization in allatectomized and *Black* mutant *Manduca sexta* larvae. J Insect Physiol. 30: 597-599.
28. Truman, J.W. (1996). Ecdysis control sheds another layer. Science. 271: 40-41.
29. Zitnan, D., Kim, Y.J., Zitnanova, I., Roller, L., et al. (2007). Complex steroid-peptide-receptor cascade controls insect ecdysis. Gen Comp Endocrinol. 153: 88-96.
30. Zitnan, D. and Adams, M.E. (2005). Neuroendocrine regulation of insect ecdysis. In. Comprehensive Molecular Insect Science, Vol. 3.
31. Zitnanova, I., Adams, M.E. and Zitnan, D. (2001). Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*. J Exp Biol. 204: 3483-3495.

32. Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., et al. (1999). Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. Neuron. 23: 523-535.
33. Gauthier, S.A. and Hewes, R.S. (2006). Transcriptional regulation of neuropeptide and peptide hormone expression by the Drosophila *dimmed* and *cryptocephal* genes. J Exp Biol. 209: 1803-1815.
34. Hewes, R.S., Schaefer, A.M. and Taghert, P.H. (2000). The cryptocephal gene (ATF4) encodes multiple basic-leucine zipper proteins controlling molting and metamorphosis in Drosophila. Genetics. 155: 1711-1723.
35. Kingan, T.G. and Adams, M.E. (2000). Ecdysteroids regulate secretory competence in Inka cells. J Exp Biol. 203: 3011-3018.
36. Park, Y., Zitnan, D., Gill, S.S. and Adams, M.E. (1999). Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*. FEBS Lett. 463: 133-138.
37. Kim, Y.J., Zitnan, D., Galizia, C.G., Cho, K.H., et al. (2006). A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol. 16: 1395-1407.
38. Zitnan, D., Hollar, L., Spalovska, I., Takac, P., et al. (2002). Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. J Exp Biol. 205: 3459-3473.
39. Kim, Y.J., Zitnan, D., Cho, K.H., Schooley, D.A., et al. (2006). Central peptidergic ensembles associated with organization of an innate behavior. Proc Natl Acad Sci USA. 103: 14211-14216.
40. Iversen, A., Cazzamali, G., Williamson, M., Hauser, F., et al. (2002). Molecular identification of the first insect ecdysis triggering hormone receptors. Biochem Biophys Res Commun. 299: 924-931.
41. Park, Y., Kim, Y.J., Dupriez, V. and Adams, M.E. (2003). Two subtypes of ecdysis-triggering hormone receptor in *Drosophila melanogaster*. J Biol Chem. 278: 17710-17715.

42. Kim, Y.J., Spalovska-Valachova, I., Cho, K.H., Zitnanova, I., et al. (2004). Corazonin receptor signaling in ecdysis initiation. Proc Natl Acad Sci USA. 101: 6704-6709.

43. Ewer, J., Gammie, S.C. and Truman, J.W. (1997). Control of insect ecdysis by a positive-feedback endocrine system: Roles of eclosion hormone and ecdysis triggering hormone. J Exp Biol. 200: 869-881.

44. Horodyski, F.M., Ewer, J., Riddiford, L.M. and Truman, J.W. (1993). Isolation, characterization and expression of the eclosion hormone gene of *Drosophila melanogaster*. Eur J Biochem. 215: 221-228.

45. Brand, A.H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118: 401-415.

46. McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., et al. (1997). Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila. Neuron. 19: 813-823.

47. Clark, A.C., del Campo, M.L. and Ewer, J. (2004). Neuroendocrine control of larval ecdysis behavior in *Drosophila*: Complex regulation by partially redundant neuropeptides. J Neurosci. 24: 4283-4292.

48. Ewer, J. and Truman, J.W. (1996). Increases in cyclic 3', 5'-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. J Comp Neurol. 370: 330-341.

49. Baker, J.D., McNabb, S.L. and Truman, J.W. (1999). The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. J Exp Biol. 202: 3037-3048.

50. Gammie, S.C. and Truman, J.W. (1999). Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. J Exp Biol. 202: 343-352.

51. Gammie, S.C. and Truman, J.W. (1997). Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. J Neurosci. 17: 4389-4397.

52. Park, Y., Kim, Y.J. and Adams, M.E. (2002). Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc Natl Acad Sci USA. 99: 11423-11428.

53. Cazzamali, G., Hauser, F., Kobberup, S., Williamson, M., et al. (2003). Molecular identification of a Drosophila G protein-coupled receptor specific for crustacean cardioactive peptide. Biochem Biophys Res Commun. 303: 146-152.

54. Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., et al. (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila. J Neurosci. 26: 573-584.

55. Luo, C.W., Dewey, E.M., Sudo, S., Ewer, J., et al. (2005). Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. Proc Natl Acad Sci USA. 102: 2820-2825.

56. Dewey, E.M., McNabb, S.L., Ewer, J., Kuo, G.R., et al. (2004). Identification of the gene encoding *bursicon*, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr Biol. 14: 1208-1213.

57. Mendive, F.M., Van Loy, T., Claeysen, S., Poels, J., et al. (2005). Drosophila molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. FEBS Lett. 579: 2171-2176.

58. Fraenkel, G., Hsiao, C. and Seligman, M. (1966). Properties of bursicon: An insect protein hormone that controls cuticular tanning. Science. 151: 91-93.

59. Fraenkel, G. and Hsiao, C. (1962). Hormonal and nervous control of tanning in fly. Science. 138: 27-29.

60. Baker, J.D. and Truman, J.W. (2002). Mutations in the Drosophila glycoprotein hormone receptor, *rickets*, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J Exp Biol. 205: 2555-2565.

61. Kimura, K., Kodama, A., Hayasaka, Y. and Ohta, T. (2004). Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*. Development. 131: 1597-1606.

62. Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet. 24: 127-222.

63. Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965). Elucidation of rate-limiting step in norepinephrine biosynthesis in perfused guinea-pig heart. J Pharmacol Exp Ther. 148: 1-8.

64. Neckameyer, W.S. and White, K. (1993). *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. J Neurogenet. 8: 189-199.

65. Nagatsu, T., Levitt, M. and Udenfriend, S. (1964). Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem. 239: 2910-2917.

66. Birman, S., Morgan, B., Anzivino, M. and Hirsh, J. (1994). A novel and major isoform of tyrosine hydroxylase in *Drosophila* is generated by alternative RNA processing. J Biol Chem. 269: 26559-26567.

67. Friggi-Grelin, F., Iche, M. and Birman, S. (2003). Tissue-specific developmental requirements of Drosophila tyrosine hydroxylase isoforms. Genesis. 35: 260-269.

68. Dunkley, P.R., Bobrovskaya, L., Graham, M.E., von Nagy-Felsobuki, E.I., et al. (2004). Tyrosine hydroxylase phosphorylation: Regulation and consequences. J Neurochem. 91: 1025-1043.

69. Hufton, S.E., Jennings, I.G. and Cotton, R.G.H. (1995). Structure and function of the aromatic amino acid hydroxylases. Biochem J. 311: 353-366.

70. Kumer, S.C. and Vrana, K.E. (1996). Intricate regulation of tyrosine hydroxylase activity and gene expression. J Neurochem. 67: 443-462.

71. Zigmond, R.E., Schwarzschild, M.A. and Rittenhouse, A.R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annu Rev Neurosci. 12: 415-461.

72. Vie, A., Cigna, M., Toci, R. and Birman, S. (1999). Differential regulation of Drosophila tyrosine hydroxylase isoforms by dopamine binding and cAMP-dependent phosphorylation. J Biol Chem. 274: 16788-16795.

73. Neckameyer, W.S., Holt, B. and Paradowski, T.J. (2005). Biochemical conservation of recombinant Drosophila tyrosine hydroxylase with its mammalian cognates. Biochem Genet. 43: 425-443.

74. Hirsh, J. and Davidson, N. (1981). Isolation and characterization of the *Dopa decarboxylase* gene of *Drosophila melanogaster*. Mol Cell Biol. 1: 475-485.

75. Livingstone, M.S. and Tempel, B.L. (1983). Genetic dissection of monoamine neurotransmitter synthesis in Drosophila. Nature. 303: 67-70.

76. Morgan, B.A., Johnson, W.A. and Hirsh, J. (1986). Regulated splicing produces different forms of *Dopa decarboxylase* in the central nervous system and hypoderm of *Drosophila melanogaster*. EMBO J. 5: 3335-3342.

77. Wright, T.R., Bewley, G.C. and Sherald, A.F. (1976). The genetics of *Dopa decarboxylase* in *Drosophila melanogaster*. II. Isolation and characterization of Dopa-decarboxylase-deficient mutants and their relationship to the alpha-methyl-dopa-hypersensitive mutants. Genetics. 84: 287-310.

78. Kraminsky, G.P., Clark, W.C., Estelle, M.A., Gietz, R.D., et al. (1980). Induction of translatable mRNA for *Dopa decarboxylase* in *Drosophila*: An early response to ecdysterone. Proc Natl Acad Sci USA. 77: 4175-4179.

79. Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W., et al. (2002). Induction of the early-late *Ddc* gene during Drosophila metamorphosis by the ecdysone receptor. Mech Dev. 114: 95-107.

80. Chen, L., O'Keefe, S.L. and Hodgetts, R.B. (2002). Control of *Dopa decarboxylase* gene expression by the Broad-Complex during metamorphosis in Drosophila. Mech Dev. 119: 145-156.

81. DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., et al. (1991). The Drosophila *Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics. 129: 385-397.

82. Hodgetts, R.B. and O'Keefe, S.L. (2006). *Dopa decarboxylase*: A model gene-enzyme system for studying development, behavior, and systematics. Annu Rev Entomol. 51: 259-284.

83. Hodgetts, R.B., Clark, W.C., O'Keefe, S.L., Schouls, M., et al. (1995). Hormonal induction of *Dopa decarboxylase* in the epidermis of Drosophila is mediated by the Broad-Complex. Development. 121: 3913-3922.

84. Wittkopp, P.J., True, J.R. and Carroll, S.B. (2002). Reciprocal functions of the Drosophila yellow and ebony proteins in the development and evolution of pigment patterns. Development. 129: 1849-1858.

85. Walter, M.F., Black, B.C., Afshar, G., Kermabon, A.Y., et al. (1991). Temporal and spatial expression of the *yellow* gene in correlation with cuticle formation and *Dopa decarboxylase* activity in Drosophila development. Dev Biol. 147: 32-45.

86. Han, Q., Fang, J., Ding, H., Johnson, J.K., et al. (2002). Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. Biochem J. 368: 333-340.

87. Brodbeck, D., Amherd, R., Callaerts, P., Hintermann, E., et al. (1998). Molecular and biochemical characterization of the *aaNAT1 (Dat)* locus in *Drosophila melanogaster*: Differential expression of two gene products. DNA Cell Biol. 17: 621-633.

88. True, J.R., Yeh, S.D., Hovemann, B.T., Kemme, T., et al. (2005). Drosophila *tan* encodes a novel hydrolase required in pigmentation and vision. PLoS Genet. 1: e63.

89. Hovemann, B.T., Ryseck, R.P., Walldorf, U., Stortkuhl, K.F., et al. (1998). The Drosophila *ebony* gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. Gene. 221: 1-9.

90. Hodgetts, R.B. (1972). Biochemical characterization of mutants affecting the metabolism of β-alanine in Drosophila. J Insect Physiol. 18: 937-947.

91. Phillips, A.M., Smart, R., Strauss, R., Brembs, B., et al. (2005). The Drosophila *black* enigma: The molecular and behavioural characterization of the *black1* mutant allele. Gene. 351: 131-142.

92. Weber, J.P., Bolin, R.J., Hixon, M.S. and Sherald, A.F. (1992). Beta-alanine transaminase activity in *black* and *suppressor of black* mutations of *Drosophila melanogaster*. Biochim Biophys Acta. 1115: 181-186.

93. Ashida, M. and Yamazaki, H. (1990). Biochemistry of the phenoloxidase system in insects: With special referemce to its activation. In: Ohnishi, F. and Ishizaki, H. (eds.), Molting and metamorphosis. Japan Scientific Societies Press, Tokyo, pp. 239-265.

94. Lemaitre, B. and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. Annu Rev Immunol. 25: 697-743.

95. Imler, J.L. and Bulet, P. (2005). Antimicrobial peptides in Drosophila: Structures, activities and gene regulation. Chem Immunol Allergy. 86: 1-21.

96. Wicker, C., Reichhart, J.M., Hoffmann, D., Hultmark, D., et al. (1990). Insect immunity. Characterization of a Drosophila cDNA encoding a novel member of the diptericin family of immune peptides. J Biol Chem. 265: 22493-22498.

97. Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., et al. (1993). A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. J Biol Chem. 268: 14893-14897.

98. Asling, B., Dushay, M.S. and Hultmark, D. (1995). Identification of early genes in the Drosophila immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. Insect Biochem Mol Biol. 25: 511-518.

99. Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P., et al. (1994). Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin. A study in insect immunity. Eur J Biochem. 221: 201-209.

100. Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., et al. (1994). Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J Biol Chem. 269: 33159-33163.

101. Levashina, E.A., Ohresser, S., Bulet, P., Reichhart, J.M., et al. (1995). Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. Eur J Biochem. 233: 694-700.

102. Kylsten, P., Samakovlis, C. and Hultmark, D. (1990). The *cecropin* locus in Drosophila: A compact gene cluster involved in the response to infection. EMBO J. 9: 217-224.

103. Ekengren, S. and Hultmark, D. (1999). Drosophila cecropin as an antifungal agent. Insect Biochem Mol Biol. 29: 965-972.

104. Lemaitre, B., Reichhart, J.M. and Hoffmann, J.A. (1997). Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci USA. 94: 14614-14619.

105. Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., et al. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity. 13: 737-748.

106. Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., et al. (1998). A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. The EMBO journal. 17: 1217-1227.

107. Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature. 415: 389-395.

108. Mengin-Lecreulx, D. and Lemaitre, B. (2005). Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the Drosophila innate immune system. J Endotoxin Res. 11: 105-111.

109. Werner, T., Liu, G., Kang, D., Ekengren, S., et al. (2000). A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proc Natl Acad Sci USA. 97: 13772-13777.

110. Steiner, H. (2004). Peptidoglycan recognition proteins: On and off switches for innate immunity. Immunol Rev. 198: 83-96.

111. Royet, J., Reichhart, J.M. and Hoffmann, J.A. (2005). Sensing and signaling during infection in Drosophila. Curr Opin Immunol. 17: 11-17.

112. Mellroth, P., Karlsson, J. and Steiner, H. (2003). A scavenger function for a Drosophila peptidoglycan recognition protein. J Biol Chem. 278: 7059-7064.

113. Mellroth, P. and Steiner, H. (2006). PGRP-SB1: An N-acetylmuramoyl L-alanine amidase with antibacterial activity. Biochem Biophys Res Commun. 350: 994-999.

114. Zaidman-Remy, A., Herve, M., Poidevin, M., Pili-Floury, S., et al. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity. 24: 463-473.

115. Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., et al. (2003). The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol. 4: 478-484.

116. Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., et al. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity. 20: 637-649.

117. Gottar, M., Gobert, V., Michel, T., Belvin, M., et al. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature. 416: 640-644.

118. Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., et al. (2002). Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae. Proc Natl Acad Sci USA. 99: 13705-13710.

119. Takehana, A., Yano, T., Mita, S., Kotani, A., et al. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. EMBO J. 23: 4690-4700.

120. Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the Drosophila immune response to monomeric DAP-type peptidoglycan. Nat Immunol. 7: 715-723.

121. Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., et al. (2006). Downregulation of the Drosophila immune response by peptidoglycan-recognition proteins SC1 and SC2. PLoS Pathog. 2: e14.

122. Michel, T., Reichhart, J.M., Hoffmann, J.A. and Royet, J. (2001). Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature. 414: 756-759.

123. Gobert, V., Gottar, M., Matskevich, A.A., Rutschmann, S., et al. (2003). Dual activation of the Drosophila Toll pathway by two pattern recognition receptors. Science. 302: 2126-2130.

124. Pili-Floury, S., Leulier, F., Takahashi, K., Saigo, K., et al. (2004). *In vivo* RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults. J Biol Chem. 279: 12848-12853.

125. Filipe, S.R., Tomasz, A. and Ligoxygakis, P. (2005). Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway. EMBO Rep. 6: 327-333.

126. Wang, L., Weber, A.N., Atilano, M.L., Filipe, S.R., et al. (2006). Sensing of Gram-positive bacteria in Drosophila: GNBP1 is needed to process and present peptidoglycan to PGRP-SA. EMBO J. 25: 5005-5014.

127. Bischoff, V., Vignal, C., Boneca, I.G., Michel, T., et al. (2004). Function of the Drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat Immunol. 5: 1175-1180.

128. Ochiai, M. and Ashida, M. (1988). Purification of a beta-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. J Biol Chem. 263: 12056-12062.

129. Gottar, M., Gobert, V., Matskevich, A.A., Reichhart, J.M., et al. (2006). Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell. 127: 1425-1437.

130. Weber, A.N., Tauszig-Delamasure, S., Hoffmann, J.A., Lelievre, E., et al. (2003). Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. Nat Immunol. 4: 794-800.

131. Hu, X., Yagi, Y., Tanji, T., Zhou, S., et al. (2004). Multimerization and interaction of Toll and Spatzle in Drosophila. Proc Natl Acad Sci USA. 101: 9369-9374.

132. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., et al. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 86: 973-983.

133. Krem, M.M. and Di Cera, E. (2002). Evolution of enzyme cascades from embryonic development to blood coagulation. Trends Biochem Sci. 27: 67-74.

134. Belvin, M.P. and Anderson, K.V. (1996). A conserved signaling pathway: The Drosophila toll-dorsal pathway. Annu Rev Cell Dev Biol. 12: 393-416.

135. Kambris, Z., Brun, S., Jang, I.H., Nam, H.J., et al. (2006). Drosophila immunity: A large-scale *in vivo* RNAi screen identifies five serine proteases required for Toll activation. Curr Biol. 16: 808-813.

136. Jang, I.H., Chosa, N., Kim, S.H., Nam, H.J., et al. (2006). A Spatzle-processing enzyme required for toll signaling activation in Drosophila innate immunity. Dev Cell. 10: 45-55.

137. Reichhart, J.M. (2005). Tip of another iceberg: Drosophila serpins. Trends Cell Biol. 15: 659-665.

138. Ligoxygakis, P., Pelte, N., Hoffmann, J.A. and Reichhart, J.M. (2002). Activation of Drosophila Toll during fungal infection by a blood serine protease. Science. 297: 114-116.
139. Levashina, E.A., Langley, E., Green, C., Gubb, D., et al. (1999). Constitutive activation of toll-mediated antifungal defense in serpin-deficient Drosophila. Science. 285: 1917-1919.
140. Horng, T. and Medzhitov, R. (2001). Drosophila MyD88 is an adapter in the Toll signaling pathway. Proc Natl Acad Sci USA. 98: 12654-12658.
141. Tauszig-Delamasure, S., Bilak, H., Capovilla, M., Hoffmann, J.A., et al. (2002). Drosophila MyD88 is required for the response to fungal and Gram-positive bacterial infections. Nat Immunol. 3: 91-97.
142. Nicolas, E., Reichhart, J.M., Hoffmann, J.A. and Lemaitre, B. (1998). *In vivo* regulation of the IkappaB homologue cactus during the immune response of Drosophila. J Biol Chem. 273: 10463-10469.
143. Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., et al. (1993). *Dif*, a dorsal-related gene that mediates an immune response in Drosophila. Cell. 75: 753-763.
144. Lemaitre, B., Meister, M., Govind, S., Georgel, P., et al. (1995). Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. EMBO J. 14: 536-545.
145. De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., et al. (2002). The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J. 21: 2568-2579.
146. Rutschmann, S., Kilinc, A. and Ferrandon, D. (2002). Cutting edge: The toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. J Immunol. 168: 1542-1546.
147. Meng, X., Khanuja, B.S. and Ip, Y.T. (1999). Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. Genes Dev. 13: 792-797.
148. Manfruelli, P., Reichhart, J.M., Steward, R., Hoffmann, J.A., et al. (1999). A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. EMBO J. 18: 3380-3391.

149. Rutschmann, S., Jung, A.C., Hetru, C., Reichhart, J.M., et al. (2000). The Rel protein DIF mediates the antifungal but not the antibacterial host defense in Drosophila. Immunity. 12: 569-580.

150. Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., et al. (2001). Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. Dev Cell. 1: 503-514.

151. Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., et al. (1995). A recessive mutation, *immune deficiency* (*imd*), defines two distinct control pathways in the Drosophila host defense. Proc Natl Acad Sci USA. 92: 9465-9469.

152. Corbo, J.C. and Levine, M. (1996). Characterization of an immunodeficiency mutant in Drosophila. Mech Dev. 55: 211-220.

153. Levashina, E.A., Ohresser, S., Lemaitre, B. and Imler, J.L. (1998). Two distinct pathways can control expression of the gene encoding the Drosophila antimicrobial peptide metchnikowin. J Mol Biol. 278: 515-527.

154. Choe, K.M., Lee, H. and Anderson, K.V. (2005). Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proc Natl Acad Sci USA. 102: 1122-1126.

155. Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., et al. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for *E. coli*. Nature. 416: 644-648.

156. Naitza, S., Rosse, C., Kappler, C., Georgel, P., et al. (2002). The Drosophila immune defense against gram-negative infection requires the death protein dFADD. Immunity. 17: 575-581.

157. Leulier, F., Vidal, S., Saigo, K., Ueda, R., et al. (2002). Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in Drosophila adults. Curr Biol. 12: 996-1000.

158. Hu, S. and Yang, X. (2000). dFADD, a novel death domain-containing adapter protein for the Drosophila caspase DREDD. J Biol Chem. 275: 30761-30764.

159. Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., et al. (2000). The Drosophila caspase Dredd is required to resist gram-negative bacterial infection. EMBO Rep. 1: 353-358.

160. Silverman, N., Zhou, R., Erlich, R.L., Hunter, M., et al. (2003). Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J Biol Chem. 278: 48928-48934.

161. Vidal, S., Khush, R.S., Leulier, F., Tzou, P., et al. (2001). Mutations in the Drosophila *dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes Dev. 15: 1900-1912.

162. Gesellchen, V., Kuttenkeuler, D., Steckel, M., Pelte, N., et al. (2005). An RNA interference screen identifies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in Drosophila. EMBO Rep. 6: 979-984.

163. Kleino, A., Valanne, S., Ulvila, J., Kallio, J., et al. (2005). Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. EMBO J. 24: 3423-3434.

164. Leulier, F., Lhocine, N., Lemaitre, B. and Meier, P. (2006). The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection. Mol Cell Biol. 26: 7821-7831.

165. Zhuang, Z.H., Sun, L., Kong, L., Hu, J.H., et al. (2006). Drosophila TAB2 is required for the immune activation of JNK and NF-kappaB. Cell Signal. 18: 964-970.

166. Lu, Y., Wu, L.P. and Anderson, K.V. (2001). The antibacterial arm of the Drosophila innate immune response requires an IkappaB kinase. Genes Dev. 15: 104-110.

167. Rutschmann, S., Jung, A.C., Zhou, R., Silverman, N., et al. (2000). Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nat Immunol. 1: 342-347.

168. Silverman, N., Zhou, R., Stoven, S., Pandey, N., et al. (2000). A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes Dev. 14: 2461-2471.

169. Hedengren, M., Asling, B., Dushay, M.S., Ando, I., et al. (1999). Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol Cell. 4: 827-837.

170. Stoven, S., Ando, I., Kadalayil, L., Engstrom, Y., et al. (2000). Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage. EMBO Rep. 1: 347-352.

171. Stoven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., et al. (2003). Caspase-mediated processing of the Drosophila NF-kappaB factor Relish. Proc Natl Acad Sci USA. 100: 5991-5996.

172. Khush, R.S., Cornwell, W.D., Uram, J.N. and Lemaitre, B. (2002). A ubiquitin-proteasome pathway represses the Drosophila immune deficiency signaling cascade. Curr Biol. 12: 1728-1737.

173. Cha, G.H., Cho, K.S., Lee, J.H., Kim, M., et al. (2003). Discrete functions of TRAF1 and TRAF2 in *Drosophila melanogaster* mediated by c-Jun N-terminal kinase and NF-kappaB-dependent signaling pathways. Mol Cell Biol. 23: 7982-7991.

174. Foley, E. and O'Farrell, P.H. (2004). Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol. 2: E203.

175. Zhou, R., Silverman, N., Hong, M., Liao, D.S., et al. (2005). The role of ubiquitination in Drosophila innate immunity. J Biol Chem. 280: 34048-34055.

176. Yagi, Y. and Ip, Y.T. (2005). Helicase89B is a Mot1p/BTAF1 homologue that mediates an antimicrobial response in Drosophila. EMBO Rep. 6: 1088-1094.

177. Tsuda, M., Langmann, C., Harden, N. and Aigaki, T. (2005). The RING-finger scaffold protein Plenty of SH3s targets TAK1 to control immunity signalling in Drosophila. EMBO reports. 6: 1082-1087.

178. Kim, M., Lee, J.H., Lee, S.Y., Kim, E., et al. (2006). Caspar, a suppressor of antibacterial immunity in Drosophila. Proc Natl Acad Sci USA. 103: 16358-16363.

179. Primrose, D.A., Chaudhry, S., Johnson, A.G., Hrdlicka, A., et al. (2007). Interactions of DNR1 with the apoptotic machinery of *Drosophila melanogaster*. J Cell Sci. 120: 1189-1199.

180. Zimmermann, K.C., Ricci, J.E., Droin, N.M. and Green, D.R. (2002). The role of ARK in stress-induced apoptosis in Drosophila cells. J Cell Biol. 156: 1077-1087.

181. Boutros, M., Agaisse, H. and Perrimon, N. (2002). Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev Cell. 3: 711-722.

182. Glise, B., Bourbon, H. and Noselli, S. (1995). *hemipterous* encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. Cell. 83: 451-461.

183. Martin-Blanco, E. (2000). p38 MAPK signalling cascades: Ancient roles and new functions. Bioessays. 22: 637-645.

184. Kockel, L., Homsy, J.G. and Bohmann, D. (2001). Drosophila AP-1: Lessons from an invertebrate. Oncogene. 20: 2347-2364.

185. Riesgo-Escovar, J.R., Jenni, M., Fritz, A. and Hafen, E. (1996). The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. Genes Dev. 10: 2759-2768.

186. Perkins, K.K., Dailey, G.M. and Tjian, R. (1988). Novel Jun- and Fos-related proteins in Drosophila are functionally homologous to enhancer factor AP-1. EMBO J. 7: 4265-4273.

187. Zhang, K., Chaillet, J.R., Perkins, L.A., Halazonetis, T.D., et al. (1990). Drosophila homolog of the mammalian jun oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc Natl Acad Sci USA. 87: 6281-6285.

188. Delaney, J.R., Stoven, S., Uvell, H., Anderson, K.V., et al. (2006). Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways. EMBO J. 25: 3068-3077.

189. Kallio, J., Leinonen, A., Ulvila, J., Valanne, S., et al. (2005). Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. Microbes Infect. 7: 811-819.

190. Kim, L.K., Choi, U.Y., Cho, H.S., Lee, J.S., et al. (2007). Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. PLoS Biol. 5: e238.

191. Park, J.M., Brady, H., Ruocco, M.G., Sun, H., et al. (2004). Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila. Genes Dev. 18: 584-594.

192. Noselli, S. (1998). JNK signaling and morphogenesis in Drosophila. Trends Genet. 14: 33-38.

193. Adachi-Yamada, T., Fujimura-Kamada, K., Nishida, Y. and Matsumoto, K. (1999). Distortion of proximodistal information causes JNK-dependent apoptosis in Drosophila wing. Nature. 400: 166-169.

194. Sluss, H.K., Han, Z., Barrett, T., Davis, R.J., et al. (1996). A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes Dev. 10: 2745-2758.

195. Galko, M.J. and Krasnow, M.A. (2004). Cellular and genetic analysis of wound healing in Drosophila larvae. PLoS Biol. 2: E239.

196. Ramet, M., Lanot, R., Zachary, D. and Manfruelli, P. (2002). JNK signaling pathway is required for efficient wound healing in Drosophila. Dev Biol. 241: 145-156.

197. Bosch, M., Serras, F., Martin-Blanco, E. and Baguna, J. (2005). JNK signaling pathway required for wound healing in regenerating Drosophila wing imaginal discs. Dev Biol. 280: 73-86.

198. Zettervall, C.J., Anderl, I., Williams, M.J., Palmer, R., et al. (2004). A directed screen for genes involved in Drosophila blood cell activation. Proc Natl Acad Sci USA. 101: 14192-14197.

199. Bidla, G., Dushay, M.S. and Theopold, U. (2007). Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J Cell Sci. 120: 1209-1215.

200. Polaski, S., Whitney, L., Barker, B.W. and Stronach, B. (2006). Genetic analysis of slipper/mixed lineage kinase reveals requirements in multiple Jun-N-terminal kinase-dependent morphogenetic events during Drosophila development. Genetics. 174: 719-733.

201. Stronach, B. and Perrimon, N. (2002). Activation of the JNK pathway during dorsal closure in Drosophila requires the mixed lineage kinase, slipper. Genes Dev. 16: 377-387.
202. Geuking, P., Narasimamurthy, R. and Basler, K. (2005). A genetic screen targeting the tumor necrosis factor/Eiger signaling pathway: identification of Drosophila TAB2 as a functionally conserved component. Genetics. 171: 1683-1694.
203. Mace, K.A., Pearson, J.C. and McGinnis, W. (2005). An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science (New York, NY. 308: 381-385.
204. Gabay, L., Seger, R. and Shilo, B.Z. (1997). MAP kinase in situ activation atlas during Drosophila embryogenesis. Development. 124: 3535-3541.
205. Gabay, L., Seger, R. and Shilo, B.Z. (1997). In situ activation pattern of Drosophila EGF receptor pathway during development. Science (New York, NY. 277: 1103-1106.
206. Brunner, D., Oellers, N., Szabad, J., Biggs, W.H., 3rd, et al. (1994). A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell. 76: 875-888.
207. Biggs, W.H., 3rd, Zavitz, K.H., Dickson, B., van der Straten, A., et al. (1994). The Drosophila *rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway. Embo J. 13: 1628-1635.
208. Inoue, H., Tateno, M., Fujimura-Kamada, K., Takaesu, G., et al. (2001). A Drosophila MAPKKK, D-MEKK1, mediates stress responses through activation of p38 MAPK. The EMBO journal. 20: 5421-5430.
209. Fukuda, M., Gotoh, Y. and Nishida, E. (1997). Interaction of MAP kinase with MAP kinase kinase: Its possible role in the control of nucleocytoplasmic transport of MAP kinase. The EMBO journal. 16: 1901-1908.
210. Pouyssegur, J., Volmat, V. and Lenormand, P. (2002). Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Biochem Pharmacol. 64: 755-763.
211. Han, Z.S., Enslen, H., Hu, X., Meng, X., et al. (1998). A conserved p38 mitogen-activated protein kinase pathway regulates Drosophila immunity gene expression. Mol Cell Biol. 18: 3527-3539.

212. Craig, C.R., Fink, J.L., Yagi, Y., Ip, Y.T., et al. (2004). A Drosophila p38 orthologue is required for environmental stress responses. EMBO reports. 5: 1058-1063.

213. Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., et al. (1999). p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol Cell Biol. 19: 2322-2329.

214. Sano, Y., Akimaru, H., Okamura, T., Nagao, T., et al. (2005). Drosophila activating transcription factor-2 is involved in stress response via activation by p38, but not c-Jun NH(2)-terminal kinase. Mol Biol Cell. 16: 2934-2946.

215. Lamprou, I., Tsakas, S., Theodorou, G.L., Karakantza, M., et al. (2005). Uptake of LPS/E. coli/latex beads via distinct signalling pathways in medfly hemocytes: The role of MAP kinases activation and protein secretion. Biochim Biophys Acta. 1744: 1-10.

216. Suzanne, M., Irie, K., Glise, B., Agnes, F., et al. (1999). The Drosophila p38 MAPK pathway is required during oogenesis for egg asymmetric development. Genes & development. 13: 1464-1474.

217. Zhuang, Z.H., Zhou, Y., Yu, M.C., Silverman, N., et al. (2006). Regulation of Drosophila p38 activation by specific MAP2 kinase and MAP3 kinase in response to different stimuli. Cellular signalling. 18: 441-448.

218. Brun, S., Vidal, S., Spellman, P., Takahashi, K., et al. (2006). The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in Drosophila. Genes Cells. 11: 397-407.

219. Shrestha, R. and Gateff, E. (1982). Ultrastructure and cytochemistry of the cell types in the larval hematopoietic organs and hemolymph of *Drosophila melanogaster*. Dev Growth Differ. 24: 64-82.

220. Rizki, R.M. and Rizki, T.M. (1984). Selective destruction of a host blood cell type by a parasitoid wasp. Proc Natl Acad Sci USA. 81: 6154-6158.

221. Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001). Postembryonic hematopoiesis in Drosophila. Dev Biol. 230: 243-257.

222. Hartenstein, V. (2006). Blood cells and blood cell development in the animal kingdom. Annu Rev Cell Dev Biol. 22: 677-712.

223. Crozatier, M. and Meister, M. (2007). Drosophila haematopoiesis. Cell Microbiol. 9: 1117-1126.

224. Tepass, U., Fessler, L.I., Aziz, A. and Hartenstein, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development. 120: 1829-1837.

225. Franc, N.C., Dimarcq, J.L., Lagueux, M., Hoffmann, J., et al. (1996). Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity. 4: 431-443.

226. Franc, N.C., Heitzler, P., Ezekowitz, R.A. and White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science. 284: 1991-1994.

227. Sears, H.C., Kennedy, C.J. and Garrity, P.A. (2003). Macrophage-mediated corpse engulfment is required for normal Drosophila CNS morphogenesis. Development. 130: 3557-3565.

228. Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., et al. (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell. 123: 335-346.

229. Ramet, M., Pearson, A., Manfruelli, P., Li, X., et al. (2001). Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity. 15: 1027-1038.

230. Dimarcq, J.L., Imler, J.L., Lanot, R., Ezekowitz, R.A., et al. (1997). Treatment of l(2)mbn Drosophila tumorous blood cells with the steroid hormone ecdysone amplifies the inducibility of antimicrobial peptide gene expression. Insect Biochem Mol Biol. 27: 877-886.

231. Agaisse, H., Petersen, U.M., Boutros, M., Mathey-Prevot, B., et al. (2003). Signaling role of hemocytes in Drosophila JAK/STAT-dependent response to septic injury. Dev Cell. 5: 441-450.

232. Irving, P., Ubeda, J.M., Doucet, D., Troxler, L., et al. (2005). New insights into Drosophila larval haemocyte functions through genome-wide analysis. Cell Microbiol. 7: 335-350.

233. Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000). Specification of Drosophila hematopoietic lineage by conserved transcription factors. Science. 288: 146-149.

234. Ashida, M. and Brey, P.T. (1997). Recent advances in research on the insect prophenol-oxidase cascade. In: Brey, P.T. and Hultmark, D. (eds.), Molecular Mechanisms of Immune Response in Insects. Chapman and Hall, London, pp. 135-172.

235. Crozatier, M., Ubeda, J.M., Vincent, A. and Meister, M. (2004). Cellular immune response to parasitization in Drosophila requires the EBF orthologue collier. PLoS Biol. 2: E196.

236. Sorrentino, R.P., Carton, Y. and Govind, S. (2002). Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated. Dev Biol. 243: 65-80.

237. Russo, J., Dupas, S., Frey, F., Carton, Y., et al. (1996). Insect immunity: Early events in the encapsulation process of parasitoid (*Leptopilina boulardi*) eggs in resistant and susceptible strains of Drosophila. Parasitology. 112 (Pt 1): 135-142.

238. Nappi, A.J. and Vass, E. (1993). Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. Pigment Cell Res. 6: 117-126.

239. Nappi, A.J., Carton, Y. and Vass, E. (1992). Reduced cellular immune competence of a temperature-sensitive *Dopa decarboxylase* mutant strain of *Drosophila melanogaster* against the parasite *Leptopilina boulardi*. Comp Biochem Physiol B. 101: 453-460.

240. Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., et al. (2004). Isolation and characterization of hemolymph clotting factors in *Drosophila melanogaster* by a pullout method. Curr Biol. 14: 625-629.

241. Bidla, G., Lindgren, M., Theopold, U. and Dushay, M.S. (2005). Hemolymph coagulation and phenoloxidase in Drosophila larvae. Dev Comp Immunol. 29: 669-679.

242. Karlsson, C., Korayem, A.M., Scherfer, C., Loseva, O., et al. (2004). Proteomic analysis of the Drosophila larval hemolymph clot. J Biol Chem. 279: 52033-52041.

243. Rizki, T.M., Rizki, R.M. and Bellotti, R.A. (1985). Genetics of a Drosophila phenoloxidase. Mol Gen Genet. 201: 7-13.

244. Meister, M. (2004). Blood cells of Drosophila: Cell lineages and role in host defence. Curr Opin Immunol. 16: 10-15.

245. Tang, H., Kambris, Z., Lemaitre, B. and Hashimoto, C. (2006). Two proteases defining a melanization cascade in the immune system of Drosophila. J Biol Chem. 281: 28097-28104.

246. De Gregorio, E., Spellman, P.T., Rubin, G.M. and Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA. 98: 12590-12595.

247. Irving, P., Troxler, L., Heuer, T.S., Belvin, M., et al. (2001). A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci USA. 98: 15119-15124.

248. Leclerc, V., Pelte, N., El Chamy, L., Martinelli, C., et al. (2006). Prophenoloxidase activation is not required for survival to microbial infections in Drosophila. EMBO Rep. 7: 231-235.

249. Sugumaran, M. (2002). Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res. 15: 2-9.

250. Lemaitre, B. and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. Annu Rev Immunol. 25: 697-743.

251. Nappi, A.J. and Ottaviani, E. (2000). Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays. 22: 469-480.

252. Kim, M.H., Joo, C.H., Cho, M.Y., Kwon, T.H., et al. (2000). Bacterial-injection-induced syntheses of N-beta-alanyldopamine and Dopa decarboxylase in the hemolymph of coleopteran insect, *Tenebrio molitor* larvae. Eur J Biochem. 267: 2599-2608.

253. Schachter, J., Perez, M.M. and Quesada-Allue, L.A. (2007). The role of N-beta-alanyldopamine synthase in the innate immune response of two insects. J Insect Physiol. 53: 1188-1197.

Chapter 2: A neuropeptide hormone cascade controls the precise onset of post-eclosion cuticular tanning in *Drosophila melanogaster*

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Introduction

Hormones play a central role in regulating temporal and spatial patterns of gene expression during the development of multicellular organisms. In the fruitfly, *Drosophila melanogaster*, the steroid hormone 20-hydroxy ecdysone (hereafter referred to as ecdysone) controls all developmental transitions as the organism develops from a fertilized egg to an adult fly (1). Ecdysone is released prior to the larval moults, pupation, and eclosion and initiates a hormonal cascade that ultimately leads to shedding of the cuticle, a process known as ecdysis.

The precise timing of ecdysis occurs in response to the neuronal release of peptide hormones, which act on ecdysone-primed tissues (Figure 2.1). In *Manduca sexta*, if ecdysone titres do not decline, ecdysis is blocked (2), suggesting that a decline in steroid hormone titre signifies the organism's readiness to shed its cuticle (3). The first hormone to be released in response to decreasing ecdysone levels is ecdysis-triggering hormone (ETH) (4). This triggers an increase in eclosion hormone (EH) levels (5,6) and, in fact, EH acts in a positive feedback loop to cause further release of ETH (7-9). Together ETH and EH are responsible for the preparatory behaviour called pre-ecdysis, which involves activities such as absorption of moulting fluid and tracheal air filling (10). EH acts via accumulation of the second messenger cGMP (7-9,11) to cause the release of crustacean cardio-active peptide (CCAP) (12,13). CCAP is thought to control the ecdysis motor response by shutting off pre-ecdysis and turning on ecdysis behaviours such as the expansion of the body to exert traction on the inside of the pupal case and the abdominal contractions that cause the emergence of the organism (6,14,15). In fact, the timing and organization of ecdysis are severely disrupted in organisms carrying CCAP ablation knockouts (14). CCAP causes activation of protein kinase A (PKA) to initiate ecdysis behaviour (16). The post eclosion hormone, Bursicon, a heterodimeric neuropeptide whose α and β subunits are encoded by the *bursicon* (*burs*) and *partner of bursicon* (*pburs*; CG15284) genes respectively, co-localizes with most CCAP neurons (16-19). Bursicon is released into the haemolymph following eclosion and acts through its

receptor, encoded by *rickets (rk)* (17-20). RK is a G-coupled hormone receptor and its activation causes an increase in cAMP (21). Wing expansion, and likely cuticular expansion and tanning, occur in response to cAMP activation of PKA.

Wing and cuticle expansion are complete within 20 minutes of eclosion and tanning of the cuticle takes 3 hours to occur. Neck-ligation of flies at eclosion prevents tanning, while flies neck-ligated 30 minutes after eclosion tan normally (22). Bursicon is normally released within 20 minutes of eclosion, and this process is disrupted in flies neck-ligated at eclosion (23). Tanning in such flies is rescued by the injection of 8-Br-cAMP or haemolymph extracted from flies 20 minutes after eclosion (18-20,23). We are interested in how the precise timing of the developmental events following eclosion is controlled by the ecdysis neuropeptide cascade.

Flies without functional EH or CCAP (EH-KO and CCAP-KO respectively) can be generated by ablating cells that produce these neuropeptides. This is achieved by triggering cell-specific apoptosis using the Gal4/UAS system to express the apoptosis gene, *reaper*, under the control of an EH or CCAP driver. EH-KO and CCAP-KO flies have a similar adult mutant phenotype to *burs* and *rk* mutants (14,17,20,24). Such adults fail to expand their wings following eclosion and exhibit a delay in tanning of their cuticle. This phenotype is mirrored in organisms that express a dominant negative form of the ecdysone receptor under the control of an EH driver, demonstrating the importance of ecdysone as a trigger for this hormonal cascade (25). Unlike *burs* and *rk* mutants, EH-KO and CCAP-KO flies also have severe defects at the larval and pupal ecdyses that result in reduced viability compared to wild type organisms (14,15,20,24).

The failure of EH-KO, CCAP-KO, *burs* and *rk* flies to properly undergo post-eclosion wing expansion and tanning indicates a loss of signalling that controls these developmental events. Tanning commonly refers to the colouration that accompanies the hardening of the cuticle. In *Drosophila melanogaster*, the mature cuticle is light brown over most of the organism, and therefore appropriately described as tanned, but the abdominal tergites are black where melanisation occurs. Since all the mutants we are aware of that reduce overall colouration also reduce the black pigmentation in the tergites, we choose to include both processes in our use of the word tanning. Tanning requires metabolites of dopamine (DA) (26). The first step in the synthesis of DA is the

rate limiting conversion of tyrosine to dihydroxyphenylalanine (Dopa) by tyrosine hydroxylase (TH), which is encoded by the *pale* (*ple*) locus (Figure 2.2A) (27-29). The conversion of Dopa to DA is then catalyzed by Dopa decarboxylase (DDC) (30), encoded by *Ddc* (31). Similar to vertebrates, dopamine plays a critical role in the CNS; however, only 6% of DDC activity is present in the brain of third instar larvae (32). The remaining activity is present in the epidermis, demonstrating the importance of epidermal DA production. In addition to the requirement for DA following eclosion, DA is needed for the rapid tanning of the puparium at pupariation and for pigmentation of the pharate adult bristles and epidermis prior to eclosion.

Both *Ddc* and *ple* mRNAs are alternatively spliced to produce neural and epidermal specific transcripts in *Drosophila* (Figure 2.2B) (33,34). The *Ddc* epidermal transcript lacks the neural-specific second exon, while the *ple* epidermal transcript contains the third and fourth exons, which are not included in the mRNA for the neural form of the enzyme. Both *Ddc* and *ple* null mutants are homozygous lethal, and rare *Ddc* mutant “escaper” flies exhibit defects in cuticular tanning and die within 24 hours of eclosion (29,35). Using transgenes to supply the respective epidermal, but not neural, forms of the enzymes to *Ddc* mutants (34) or to *ple* mutants (36) rescues their lethality, illustrating the importance of epidermal DA production in fly development. In vertebrates, TH activity is regulated at a variety of levels, including transcriptional regulation, alternative splicing, RNA stability, DA feedback inhibition, and protein kinase activation (37-40). Both transcriptional regulation and alternative splicing regulate TH activity in *Drosophila* (33). The additional exons included in the epidermal transcript of *ple* encode a very acidic segment of 71 amino acids inserted into the regulatory domain of this enzyme that reduces the sensitivity of TH to DA inhibition activity *in vitro* (41). Furthermore, *Drosophila* TH is activated, *in vitro*, by phosphorylation at Ser32, a site homologous to vertebrate Ser40 (41), the major site of PKA phosphorylation and activation (39,40). Phosphorylation of Ser32 has been proposed to increase the activity of the epidermal isoform of TH by reducing the ability of the protein to bind to DA, which inhibits its activity, and by increasing the rate of binding of the essential cofactor tetrahydrobiopterin (41). Interestingly, both CCAP and

Bursicon activate PKA by elevating cAMP levels (16,20), suggesting that either of these neuropeptides may be involved in the activation of TH to regulate the onset of tanning.

In this paper, we set out to determine how the ecdysis neuropeptide hormone cascade regulates the precise timing of developmental events following eclosion. To achieve this, we focused on the process of tanning because its biochemical basis is known and it is a readily scored marker of post-eclosion development. We find that the onset of tanning does not depend on the regulation of *Ddc*. Rather, it is the regulation of TH protein accumulation and activity, elicited by the ecdysis neuropeptide hormone cascade that precisely controls tanning. Specifically, post-eclosion accumulation of TH from the *ple* transcripts that are already present in pharate adults is regulated by CCAP, while TH is activated by PKA as a result of Bursicon signalling through RK. Regulation of TH transcription plays no part in the switch, effected by the ecdysis hormone cascade, that triggers post-eclosion tanning.

Results

Both *ple* and *Ddc* transcripts accumulate prior to eclosion

To determine whether control of tanning is regulated at a transcriptional level, we examined the transcriptional profile of epidermal *ple* and *Ddc* after pupariation by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Figure 2.3). For each indicated time point, isolated RNA was co-reverse transcribed with a *ple* or *Ddc* specific primer and an *RpL32* specific primer to serve as a loading control. For *ple*, the forward amplification primer lies within the first exon, and the reverse primer is anchored within the epidermal specific third exon (Figure 2.2B), so the product represents only the epidermal transcript. The *Ddc* forward and reverse primers are anchored in the first and fourth exons respectively, which are common to both epidermal and neural transcripts (Figure 2.2B); however, the PCR conditions are such that we only amplify the shorter epidermal transcript. A number of *yw* white pre-pupae (WPP) were collected and sampled every 12 hours until 156 hours after puparium formation (APF), approximately 2 days after eclosion occurs. Under our culture conditions, eclosion occurred, on average, at 108 hours APF. At this time, approximately half the flies had eclosed from the sample collected as WPP. Both pharate adults and newly eclosed flies collected at 108 hours APF were combined for RNA extraction, as we see no difference in *ple* or *Ddc* transcript

levels in these distinct populations (data not shown). Both *ple* and *Ddc* transcript levels are high in WPP. After this stage, the profiles of transcription of each gene differ substantially: *ple* transcript levels drop to low levels within 12 hours of pupariation, and remain low until 72 hours APF when they rise dramatically (Figure 2.3A). Transcript levels remain high until 120 hours APF, approximately 12 hours after eclosion. *Ddc* transcript levels fall more slowly after pupariation (Figure 2.3B), reaching minimal levels 24 hours APF. Levels begin to rise 60 hours APF, a full 48 hours prior to eclosion, peak 24 hours before eclosion and decrease thereafter.

TH protein and activity levels are high in pharate adults, drop before eclosion, and rise thereafter

Transcriptional control of *ple* and *Ddc* cannot regulate the precise timing of tanning following eclosion since transcripts of both genes accumulate prior to eclosion. DDC has never been shown to be regulated at a translational or post-translational level, and high levels of the active enzyme are present at eclosion (42). However, TH is known to be under both translational and activational control in mammalian systems (39,40). For this reason, we looked at TH protein accumulation from pupariation until after eclosion (Figure 2.4A). We staged flies as WPP and sampled the collection every 12 hours. In addition, we separated the 108 hour APF organisms into pharate adult and eclosed adults, in order to examine the differences in these distinct populations. The TH antibody is not epidermal-specific; however, identification of the epidermal isoform of TH is easily facilitated as it migrates much slower than the neural isoform. To simplify, only the epidermal isoform of TH is shown. The high level of TH in WPP decreases to an undetectable amount by 12 hours APF, and rises again 84 hours APF (Figure 2.4A). Protein levels drop prior to eclosion but rise sharply thereafter, remaining high for about 12 hours.

We also examined the activity profile of TH in *yw* organisms (Figure 2.4B). We find that TH activity (expressed as a percentage of WPP activity) closely parallels the protein profile of TH. Enzyme activity can still be detected 132 hours APF although protein levels are below the limits of detection by this time.

TH protein and activity levels are severely reduced after eclosion in CCAP-KO flies but not EH-KO, *burs*^{Z1091}, and *rk*^{4} mutants

EH-KO, CCAP-KO, *burs*, and *rk* flies show defects in tanning at eclosion. To determine whether the ecdysis neuropeptide signalling pathway (Figure 2.1) plays a role in the regulation of TH at eclosion, we examined the transcriptional, translational, and activity profiles of TH in these flies (Figure 2.4C, 2.4D). Organisms were staged as WPP and sampled every 24 hours until 144 hours APF. Two samples were collected at the 108 hour time point comprising pharate adults and eclosed adults. No CCAP-KO flies eclose by 108 hours, so this time point is absent in Figure 2.4C. In the original description of EH-KO flies, it was noted that the proportion of flies that failed to expand their wings varied depending on the UAS-*rpr* line used (43). Under our conditions, 58% (n=746) of the flies failed to expand their wings fully. For the experiments shown, only flies that failed to expand their wings were included in the post-eclosion analyses. For most of our analysis, we used *burs*^{Z1091}and *rk*^{4} homozygous flies, although we did some complementary heteroallelic studies using the *burs*^{Z5569} and *rk*^{1} alleles. While *rk*^{1} and *rk*^{4} mutations are null mutants due to a truncation of the receptor in the critical transmembrane domain (20), both *burs*^{Z1091} and *burs*^{Z5569} mutations likely cause hypomorphic mutations due to single amino acid conversions that fail to completely eliminate protein function (17). *ple* transcript levels in the EH-KO, CCAP-KO, *burs*^{Z1091} and *rk*^{4} flies mimicked those exhibited by the *yw* control flies (data not shown). TH protein levels in EH-KO, *burs*^{Z1091} and *rk*^{4} flies are similar to those in the *yw* control organisms (compare Figure 2.4C to Figure 2.4A). In the mutants and ablation knock-out flies, TH protein levels are high in WPP, decreasing to undetectable levels in the next 24-48 hours. Protein accumulates again at 96 hours but drops once more in all four lines just prior to eclosion (see Figure 2.4C, 108 hour pharate adults). Protein appears following eclosion in EH-KO, *burs*^{Z1091} and *rk*^{4} flies and detectable levels persist longer than in the control flies. Importantly, TH protein fails to accumulate in CCAP-KO flies following eclosion.

TH activity measurements were also carried out in EH-KO, CCAP-KO, *burs*^{Z1091} and *rk*^{4} flies (Figure 2.4D). All four mutants show relatively normal profiles of enzyme activity during metamorphosis. EH-KO, *burs*^{Z1091} and *rk*^{4} flies exhibit the rapid increase

in activity normally seen immediately after eclosion. In contrast, CCAP-KO flies show severely reduced TH activity following eclosion compared to *yw* flies.

TH protein is translated within an hour of eclosion and transiently phosphorylated and activated thereafter

Two lines of evidence suggest that TH protein accumulation following eclosion is regulated: 1) TH protein levels drop near eclosion in control flies (Figure 2.4A) and rise again following eclosion, while *ple* transcript levels do not fluctuate during this period (Figure 2.3A); 2) TH protein is absent after eclosion in CCAP-KO flies (Figure 2.4C), although *ple* is transcribed normally (data not shown), indicating that CCAP may be required for TH accumulation following eclosion. To determine precisely when TH protein first appears, Western blotting was carried out on organisms collected at eclosion and every 30 minutes thereafter (top line, Figure 2.5A). We find that TH is absent in flies at eclosion, and is first detectable 1 hour later. Protein levels reach a maximum 2 hours after eclosion, and remain elevated until at least 12 hours after eclosion (Figure 2.4A). We attribute the presence of TH in the 108 hour sample of eclosed flies in Figure 2.4A to organisms older than 1 hour.

The major site of activational control of Drosophila TH is the PKA phosphorylation site at Ser32 (41), and both CCAP and RK signalling lead to activation of PKA (16,21). The Ser32 residue is homologous the rat TH Ser40 residue, so we were able to employ a monoclonal antibody specific to the rat phospho-Ser40-TH to determine the phosphorylation state of Ser32 (bottom line, Figure 2.5A) after eclosion. Phosphorylated TH is detected only during a very short interval 1.5 to 3 hours after eclosion.

We also examined TH activity following eclosion. Activity is low in flies immediately following eclosion, and begins to increase 1.5 hours after eclosion, peaking at 2.5 hours. This peak of activity corresponds to the time of phosphorylation and tanning.

TH phosphorylation and activity are eliminated in flies neck-ligated at eclosion

Flies neck-ligated at eclosion fail to tan rapidly thereafter, while those neck-ligated 30 minutes after eclosion tan normally (22). The lack of tanning is due to the prevention of Bursicon release (18-20,23) and can be rescued in neck-ligated flies by

injection of 8-Br-cAMP (20), a membrane permeable analog of cAMP. We sought to determine if the rescue by 8-Br-cAMP injection was a result of the activation of the epidermal form of TH. Flies were neck-ligated at or 30 minutes following eclosion, and those treated with 8-Br-cAMP were injected immediately. All flies were aged a further 2.5 hours before TH Western blots and activity measurements were carried out (Figure 2.6A). We find that no TH protein is present at eclosion in control flies, in agreement with Figure 2.5A, but protein is detectable 2.5 hours later. In the flies ligated at eclosion, less protein is apparent than in control flies, and phosphorylation of Ser32 does not occur (Figure 2.6A). When flies are neck-ligated 30 minutes after eclosion, they accumulate and phosphorylate TH. TH phosphorylation can be restored in flies neck-ligated at eclosion by injection of 8-Br-cAMP.

Neck-ligation of flies at eclosion reduces TH activity to 6% of the level in control flies (Figure 2.6B). Flies neck-ligated 30 minutes after eclosion have double the activity of those neck-ligated at eclosion, and flies injected with 8-Br-cAMP have nearly 6-fold higher TH activity than the flies neck-ligated at eclosion.

Injection of 8-Br-cAMP rescues translation, phosphorylation, and activity defects in EH-KO, CCAP-KO, *burs* and *rk* flies

The absence of TH following eclosion in CCAP-KO flies (Figure 2.4C), although *ple* is transcribed normally, and the tanning defects in EH-KO, CCAP-KO, *burs* and *rk* mutants prompted us to examine whether the injection of 8-Br-cAMP affected TH levels and phosphorylation states in these mutants (Figure 2.7A). We also injected CCAP-KO flies with CCAP in an attempt to rescue the defect in TH accumulation. Flies were collected 2.5 hours after eclosion, a time when TH protein, phosphorylation and activity levels are high in control organisms (Figure 2.5). When EH-KO, CCAP-KO, and rk^4 mutant flies were injected with 8-Br-cAMP, tanning of the adult cuticle occurred, in contrast to un-injected controls ((20); and data not shown). We find that TH is absent in EH-KO and CCAP-KO flies, and consequently no phosphorylated protein is detected (Figure 2.7A, lanes 2 and 4). This absence of TH in EH-KO flies appears to contradict the accumulation of TH evident in the 108 hour time point shown in Figure 2.4C. We assume the 108 hour sample included organisms older than 2.5 hours. TH levels and phosphorylation are restored in both EH-KO and CCAP-KO flies injected with 8-Br-

cAMP (Figure 2.7A, lanes 3 and 5), and injection of CCAP into CCAP-KO flies causes accumulation, but not phosphorylation, of TH (Figure 2.8). Both *burs*^{Z1091} and *rk*^{4}flies have normal levels of TH protein (Figure 2.7A, lanes 6 and 8), but Ser32 phosphorylation is reduced in *burs*^{Z0191}and absent in *rk*^{4}flies. We ruled out second site mutation effects in the homozygous lines by showing that TH protein levels were normal in *burs*^{Z1091}*/burs*^{Z5569} *and rk*^{1}*/rk*^{4} heterozygotes, although Ser32 phosphorylation was absent (Figure 2.9). The phosphorylation defect in *burs*^{Z1091} and *rk*^{4} mutants is rescued by 8-Br-cAMP injection (Figure 2.7A, lanes 7 and 9).

Loss of TH protein in EH-KO and CCAP-KO flies corresponds with a loss of activity (Figure 2.7B). Injection of 8-Br-cAMP restores TH activity levels in these flies. Activity is only slightly reduced in *burs*^{Z1091} mutants and unchanged by injection of 8-Br-cAMP. The reduced activity in *rk*^{4} flies is restored by 8-Br-cAMP injection.

Discussion

In Drosophila, the onset of tanning of the puparium occurs within one hour after the wandering larva becomes sessile. This requires metabolites of DA, the production of which is dependent on the action TH and DDC. Transcripts levels of both genes, and TH protein and activity levels, are all high in WPP (Figures 3, 4). Unlike at eclosion, TH does not appear to be activated by PKA phosphorylation at pupariation for the rapid tanning of the pupal case (data not shown). This is not unexpected since the ecdysis neuropeptides are not released until a full 12 hours after puparium formation. During the late third instar, the relatively insoluble tyrosine, which is indispensible for tanning, is stored as a more soluble derivative, tyrosine-*O*-phosphate (Tyr-P) (See Appendix 1) (44). Tanning at pupariation is likely controlled by the release of tyrosine from Tyr-P. No appreciable accumulation of Tyr-P occurs prior to eclosion (44,45), suggesting that post-eclosion tanning is switched on by a different mechanism.

We established a role for the ecdysis neuropeptide cascade in post-eclosion tanning by examining the regulation of two genes, *ple* and *Ddc*, which encode two enzymes with critical roles in tanning. We used semi-quantitative RT-PCR to examine the profile of transcription after puparium formation. Levels of both transcripts are high in WPP (Figure 2.3), but they drop and then rise again prior to eclosion. *Ddc* levels begin to increase 60 hours APF, reach their peak 84 hours APF, and decline thereafter. DDC

enzyme activity is required prior to eclosion for pigmentation of the pharate adult bristles and epidermis and after eclosion for tanning of the adult cuticle, and reaches a peak at eclosion (42). This indicates that *Ddc* is transcribed and translated prior to eclosion to ensure enzyme activity is present when substrate becomes available. In this paper, we investigated whether the control of tanning, was effected by the transcriptional, translational, or post-translational regulation of TH.

Levels of *ple* transcripts are high during the 24 hour period spanning eclosion (Figure 2.3A). The early appearance of *ple* transcripts is not surprising, as pigmentation of the pharate adult bristles and epidermis occurs between 84 and 96 hours APF. Both *ple* (and *Ddc*) transcription are normal in EH-KO, CCAP-KO, *burs*^{Z1091} and *rk*^{4} flies (data not shown). The accumulation of *ple* transcripts prior to eclosion, the maintenance of high levels of TH transcription until 12 hours after eclosion, and the fact that neuropeptide mutant and ablation knock-out flies exhibit normal *ple* transcription, led us to conclude that the precise onset of tanning following eclosion is not due to regulation of *ple* transcription.

TH protein and activity levels are high prior to eclosion when pigmentation of the pharate adult bristles and epidermis occurs (Figure 2.4). Levels fall rapidly just prior to eclosion and rise thereafter. During this entire time, *ple* transcripts are present, suggesting that protein levels are being regulated. The drop in TH protein levels may occur through repression of translation from *ple* transcripts and/or increased turnover of the protein. The complete failure of CCAP-KO flies to accumulate TH protein following eclosion (Figure 2.4C), although they transcribe *ple* normally (data not shown), indicates a role for CCAP in this process. This could occur at the level of translation; alternatively, CCAP signalling may alter TH protein stabilization. We believe that since PKA signalling has been shown to regulate proteins involved in translational control (46), it is more likely that CCAP signalling activates PKA to cause translation, not stabilization, of TH following eclosion.

EH-KO, *burs*^{Z1091}, and *rk*^{4} flies all appear to have relatively normal TH protein and activity profiles (compare Figures 2.4C and 2.4A and Figure 2.4D to 2.4B). While all three exhibit a considerable range of activity in WPP, the pupal cases of these organisms tan normally. Despite the initial delay in TH accumulation in EH-KO flies

following eclosion (Figure 2.7A), these flies, and *burs*^{Z1091} and *rk*^{4} mutants all maintain high levels of TH until 144 hours APF, a time when control flies no longer have TH protein (Figure 2.4A). The persistence of TH longer than normal indicates a developmental delay in the execution of the neuropeptide hormone cascade. Interestingly, *rk*^{4} flies also show a delay in degradation of TH following pupariation (Figure 2.4C). Perhaps there is a requirement for RK signalling to trigger TH degradation following tanning of the puparium.

Neck-ligation of flies at eclosion prevents tanning, while flies ligated 30 minutes after eclosion tan normally (22). Furthermore, tanning of flies neck-ligated at eclosion can be rescued by injection of 8-Br-cAMP (20). TH protein begins to accumulate 1 hour after eclosion in control flies (Figure 2.5A). Phosphorylation of the protein by PKA at Ser32 then leads to enzyme activity appearing between 1.5 and 3 hours after eclosion (Figure 2.5). Our results indicate that the translational and activational state of TH is responsible for controlling tanning following eclosion. We find that TH protein accumulates, albeit not to wild-type levels, but is not phosphorylated (Figure 2.6A) in flies neck-ligated at eclosion. As a result, these flies had reduced TH activity (Figure 2.6B). By interrupting neuropeptide signalling after eclosion, we have shown that the element that controls TH translation is released prior to eclosion. The loss of TH accumulation in CCAP-KO flies (Figure 2.4C and 2.7A) and the restoration of TH accumulation upon injection of CCAP (Figure 2.8) suggests that CCAP is responsible for inducing TH translation.

Flies neck-ligated 30 minutes after eclosion translate and phosphorylate TH normally (Figure 2.6A). By allowing neuropeptide signalling after eclosion, we have demonstrated that a factor is released within 30 minutes of eclosion that causes phosphorylation and therefore activation of TH. The reduced phosphorylation of Ser32 in *burs*^{Z1091} flies, and complete loss of phosphorylation in *rk*^{4}*, burs*^{Z1091}*/burs*^{Z5569}*, and rk*^{1}*/rk*^{4} flies (Figure 2.7A and 2.9) suggests that Bursicon signalling through RK controls this process. Activity levels of TH are significantly reduced in flies neck-ligated at eclosion compared to control flies (Figure 2.6B). Flies ligated 30 minutes after eclosion show 2-fold higher levels compared to flies neck-ligated at eclosion and this difference probably accounts for the presence or absence of tanning. This suggests that a critical

threshold of TH activity exists that is surpassed in the flies ligated at 30 minutes. Thus, while the activity present in these flies is significantly less than that in control flies, the organisms have sufficient TH activity to tan, while flies ligated at eclosion do not attain the threshold of activity required for tanning. Injection of 8-Br-cAMP into flies neck-ligated at eclosion rescues tanning by restoring phosphorylation and therefore activation of TH (Figure 2.6). Although injection of 8-Br-cAMP does not restore TH activity to control levels, it increases activity nearly 6-fold (Figure 2.6B), achieving the threshold of activity required for tanning following eclosion.

These results, taken together, suggest that at least two factors control the precise timing of tanning after eclosion. One, released prior to eclosion, causes translation of TH; the other, released after eclosion, causes phosphorylation and activation of TH. Both EH and CCAP are released prior to eclosion to control pre-ecdysis and ecdysis respectively (5,6,12,13). EH-KO and CCAP-KO flies both exhibit extreme post-eclosion tanning defects. EH-KO flies take more than 9 hours to tan and CCAP-KO flies fail to tan (M.M. Davis, unpublished). TH protein is undetectable in EH-KO flies immediately following eclosion (Figure 2.7A), but these flies do eventually accumulate TH (Figure 2.4C) and tan. The complete failure of CCAP-KO flies to tan, combined with the fact that CCAP-KO flies fail to accumulate TH (Figures 2.5A and 2.8A) from the *ple* transcripts that are present at eclosion (data not shown), suggest that CCAP release is responsible for inducing TH translation. The initial failure of EH-KO flies to accumulate TH (Figure 2.7A) is likely caused by a failure to trigger the rapid release of CCAP (Figure 2.1). Presumably, enough CCAP is eventually released in these flies to effect the translation of TH and eventually tanning, because the EH genetic ablation is leaky. Consistent with this prediction, EH-KO flies that expand their wings accumulate TH normally (data not shown), suggesting CCAP is released normally in these flies. TH translation is restored in CCAP-KO flies injected with CCAP (Figure 2.8) and rescue of TH accumulation and phosphorylation occurs when EH-KO and CCAP-KO flies are injected with 8-Br-cAMP (Figure 2.7). Rescue of both defects likely occurs because injection of 8-Br-cAMP activates PKA in CCAP target cells, thus circumventing the need for CCAP release (Figure 2.1), and also activates PKA in TH expressing cells, leading to phosphorylation and activation of TH.

Our data suggest that the post-eclosion factor causing the phosphorylation of Ser32 is the heterodimeric hormone Bursicon. It is responsible for tanning and wing expansion (17-19,23) and acts through its receptor RK (20). Consistent with the role of Bursicon in the phosphorylation of TH, *rk*^{4} flies have reduced Ser32 phosphorylation and activity (Figure 2.7). These flies show a delay in tanning, taking up to 9 hours to tan (20). Injection of 8-Br-cAMP rescues tanning by restoring phosphorylation and therefore activation of TH (Figure 2.7).

Two mutants in the α subunit of Bursicon have been identified, of which one, *burs*^{Z5569}, shows a delay in tanning in 40% of the progeny, whereas a delay is present in 82% of *burs*^{Z1091}/*burs*^{Z5569} flies (17). The *burs*^{Z1091} mutant does not show a delay in tanning, although phosphorylation of TH is reduced in these flies (Figure 2.7A). Phosphorylation of Ser32 is undetectable in *burs*^{Z1091}/*burs*^{Z5569} flies (Figure 2.9), likely causing the more severe tanning defect seen in these flies. The reduced phosphorylation of TH in *burs*^{Z1091} flies corresponds to a minor loss of TH activity (Figure 2.7B). Thus, it seems that the threshold TH activity required for proper tanning is achieved in *burs*^{Z1091} flies, although they do not have wild-type levels of TH phosphorylation or activity. Normal tanning in these flies cannot be attributed to residual activity of the β subunit of Bursicon, CG15284, recently proposed to be encoded by *pu* (S. McNabb and J. Truman, personal communication), as neither subunit independently confers Bursicon activity (18,19). The *burs*^{Z1091} allele is likely a hypomorph, and creation of a null allele would be useful. Additional studies on the activational state of TH in *pu* or *burs* null mutants will help elucidate why tanning is not delayed in *burs*^{Z1091} flies.

Our data indicate that CCAP is responsible for initiating TH translation following eclosion. In Drosophila, translational regulation often occurs through microRNA (miRNA)-dependent RNAi-mediated repression through binding sites in the 3'UTR of transcripts (47). Three miRNAs have been predicted to regulate TH translation in Drosophila (48). These miRNAs include let-7, which has 2 putative binding sites, and mir-iab-4-3p and mir-iab-4-5p which have one potential site each. It is conceivable that one or more of these miRNAs, in association with the RISC complex, could bind to *ple* transcripts to cause the repression of translation through a miRNA-dependent RNAi-mediated mechanism. It is also plausible that PKA, activated by CCAP signalling, may

relieve repression of TH translation by phosphorylation of one of the subunits of the RISC complex or associated proteins. Future work will establish whether there is a role for these miRNAs in the repression of TH translation prior to eclosion.

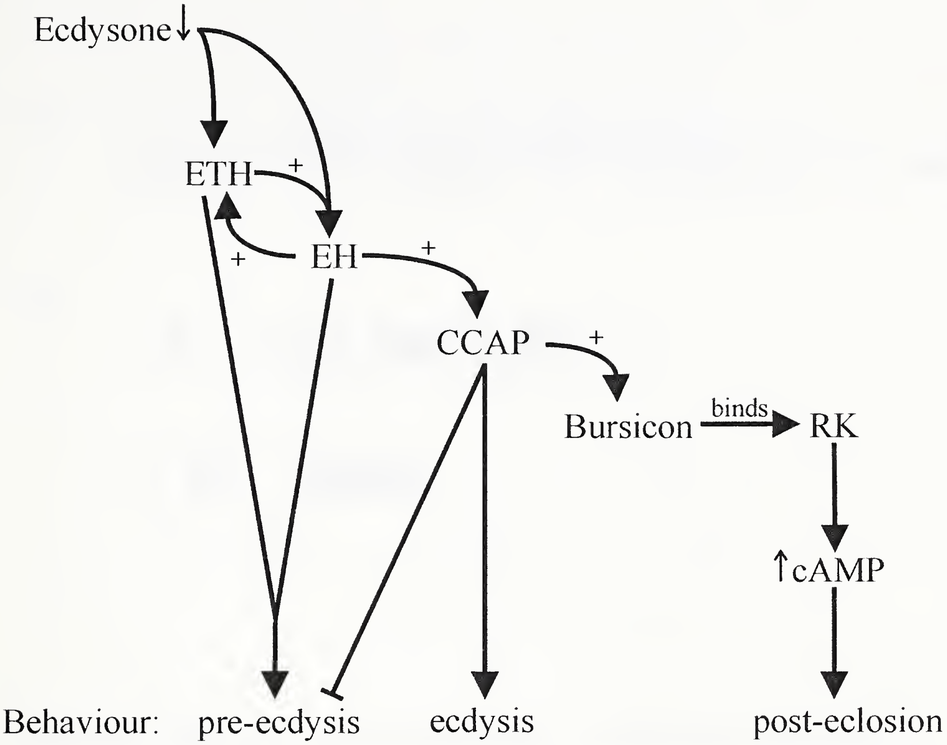

Figure 2.1: The neuropeptide signalling pathway at eclosion. In response to decreasing levels of ecdysone, ETH is released, triggering the release of EH. These hormones act together in a positive feedback loop increasing the release of one another and regulate pre-ecdysis behaviours. EH causes the release of CCAP, which shuts off pre-ecdysis and turns on the ecdysis motor program. At eclosion, CCAP causes release of Bursicon, which binds to its receptor RK, and induces post-eclosion events by elevating levels of cAMP and causing tanning. This figure is modified from (5) and (43).

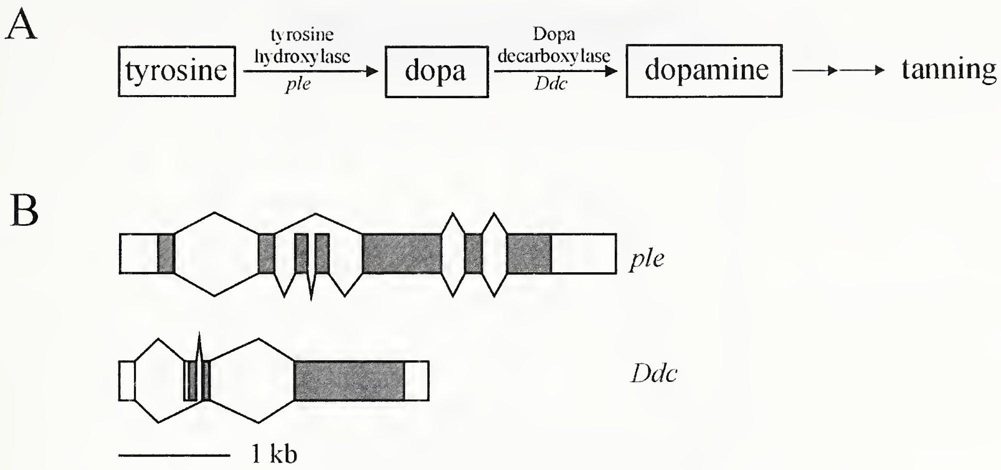

Figure 2.2: Critical steps in the tanning pathway. A. Metabolites of dopamine are required for tanning. Tyrosine hydroxylase (encoded by *ple*) converts tyrosine into Dopa and Dopa decarboxylase (encoded by *Ddc*) catalyzes the conversion of Dopa to dopamine. B. Alternative splicing of *ple* and *Ddc*. Both *ple* and *Ddc* are alternatively spliced to produce neural specific (shown above exon boxes) and epidermal specific (shown below exon boxes) transcripts. The coding sequence is shaded in grey.

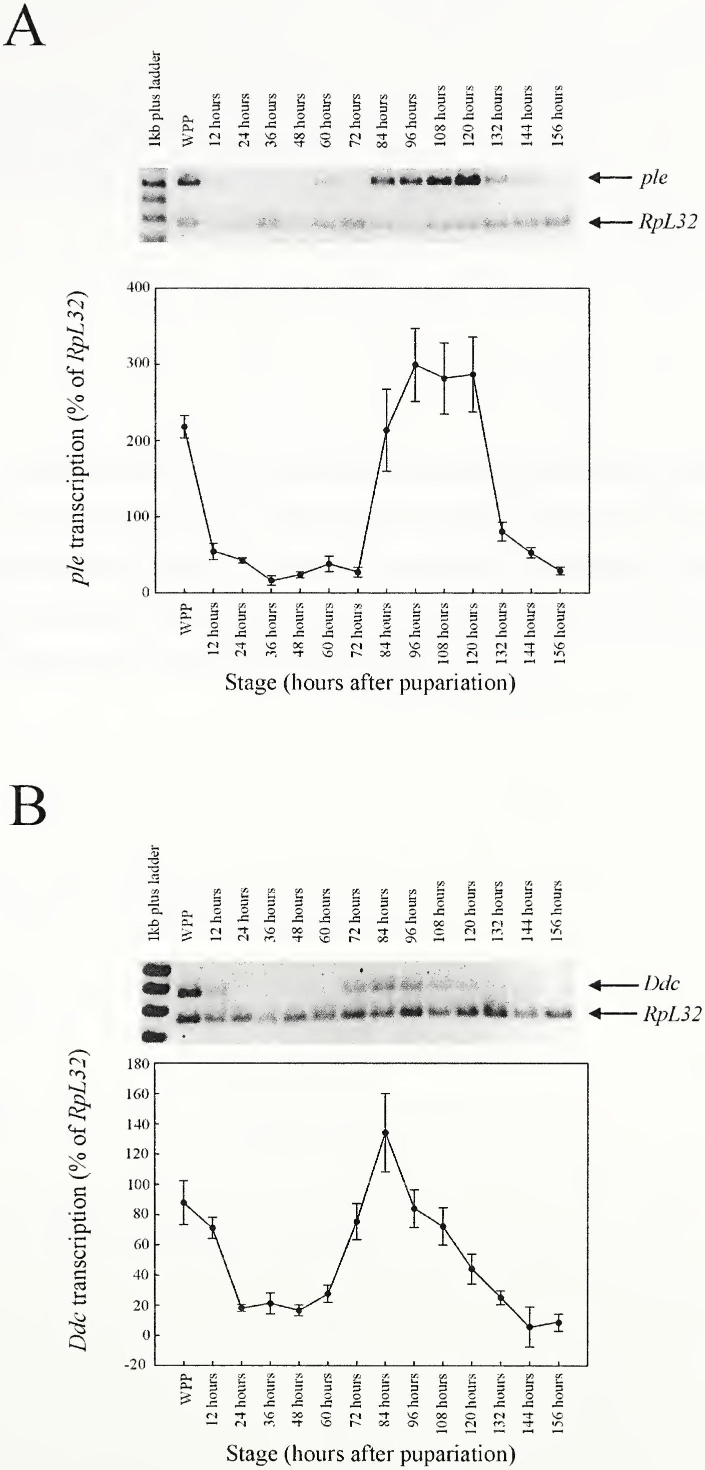
A
1kb plus ladder
WPP
12 hours
24 hours
36 hours
48 hours
60 hours
72 hours
84 hours
96 hours
108 hours
120 hours
132 hours
144 hours
156 hours
ple
RpL32
ple transcription (% of RpL32)
0
100
200
300
400
Stage (hours after pupariation)
B
Ddc
Ddc transcription (% of RpL32)
-20
0
20
40
60
80
100
120
140
160
180

Figure 2.3: Quantitative RT-PCR of *ple* and *Ddc* transcripts following pupariation. (A) *ple* or (B) *Ddc* transcripts are shown with an *Rpl32* loading control. Bands from at least four different extracts for each time point were quantified and averaged, and expressed as a percent of the loading control band to produce the graphs. Error bars in this and subsequent figures show standard error of the mean.

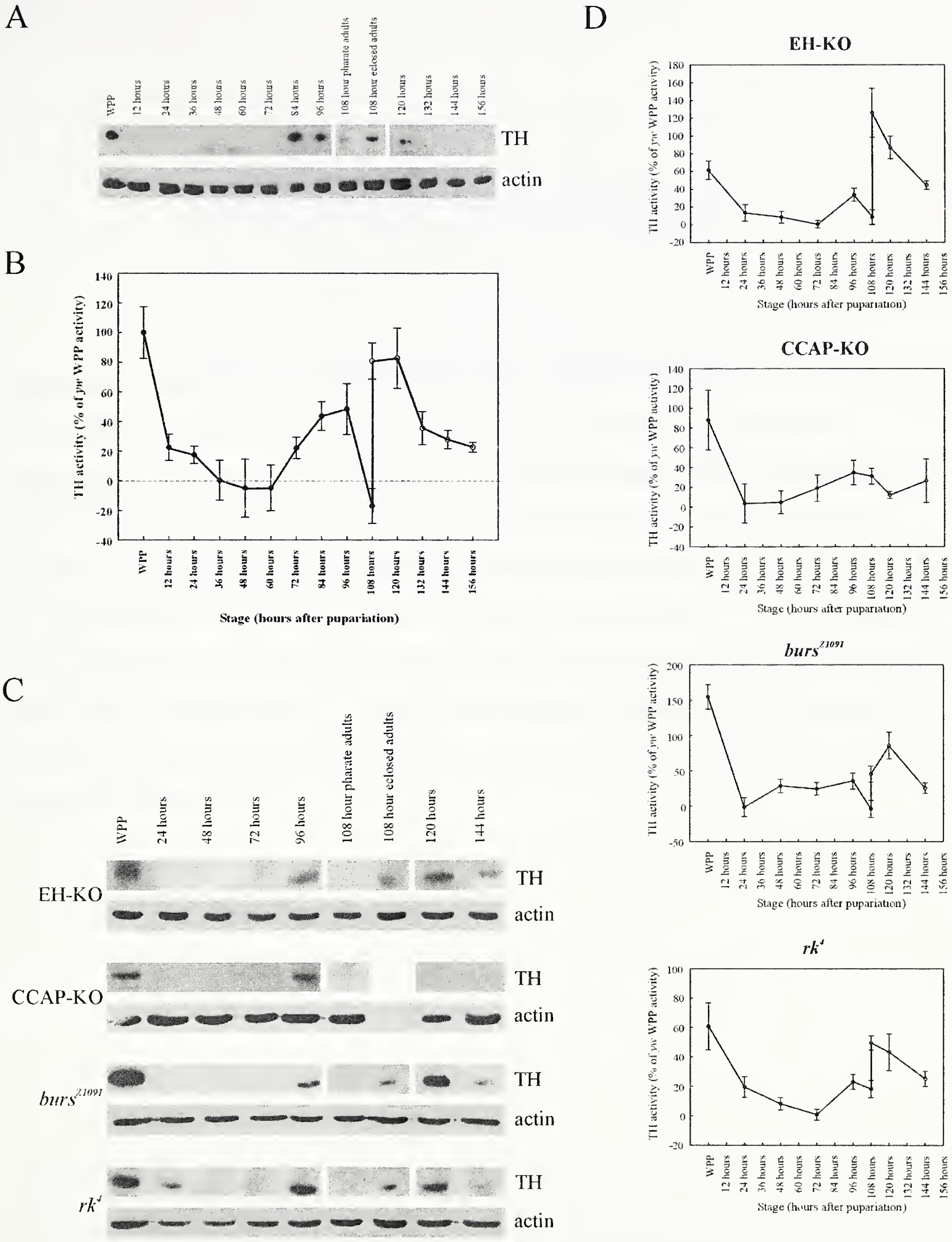
A
WPP
12 hours
24 hours
36 hours
48 hours
60 hours
72 hours
84 hours
96 hours
108 hour pharate adults
108 hour eclosed adults
120 hours
132 hours
144 hours
156 hours
TH
actin
B
TH activity (% of yw WPP activity)
140
120
100
80
60
40
20
0
-20
-40
WPP
12 hours
24 hours
36 hours
48 hours
60 hours
72 hours
84 hours
96 hours
108 hours
120 hours
132 hours
144 hours
156 hours
Stage (hours after pupariation)
C
WPP
24 hours
48 hours
72 hours
96 hours
108 hour pharate adults
108 hour eclosed adults
120 hours
144 hours
EH-KO
TH
actin
CCAP-KO
TH
actin
burs^Z1091
TH
actin
rk^4
TH
actin
D
EH-KO
TH activity (% of yw WPP activity)
180
160
140
120
100
80
60
40
20
0
-20
WPP
12 hours
24 hours
36 hours
48 hours
60 hours
72 hours
84 hours
96 hours
108 hours
120 hours
132 hours
144 hours
156 hours
Stage (hours after pupariation)
CCAP-KO
TH activity (% of yw WPP activity)
140
120
100
80
60
40
20
0
-20
-40
Stage (hours after pupariation)
burs^Z1091
TH activity (% of yw WPP activity)
200
150
100
50
0
-50
Stage (hours after pupariation)
rk^4
TH activity (% of yw WPP activity)
100
80
60
40
20
0
-20
Stage (hours after pupariation)

Figure 2.4: TH protein and activity profiles in *yw,* EH-KO, CCAP-KO, *burs*^{Z1091}, and *rk*^{4} flies following puparium formation. Times are measured from pupariation. A. Western blot of epidermal TH from *yw* flies. A blot showing an actin loading control is shown below the TH Western in this and subsequent figures. B. TH activity in *yw* organisms. TH activity in this and subsequent figures is expressed as the *yw* WPP value. C. Western blots of epidermal TH from EH-KO, CCAP-KO, *burs*^{Z1091}, and *rk*^{4} flies. D. TH activity in the same organisms expressed as a percentage of the *yw* white pre-pupae (WPP) value. In parts B and D, closed circles represent organisms within the pupal case, open circles represent adult flies and values are averaged over a minimum of three separate experiments.

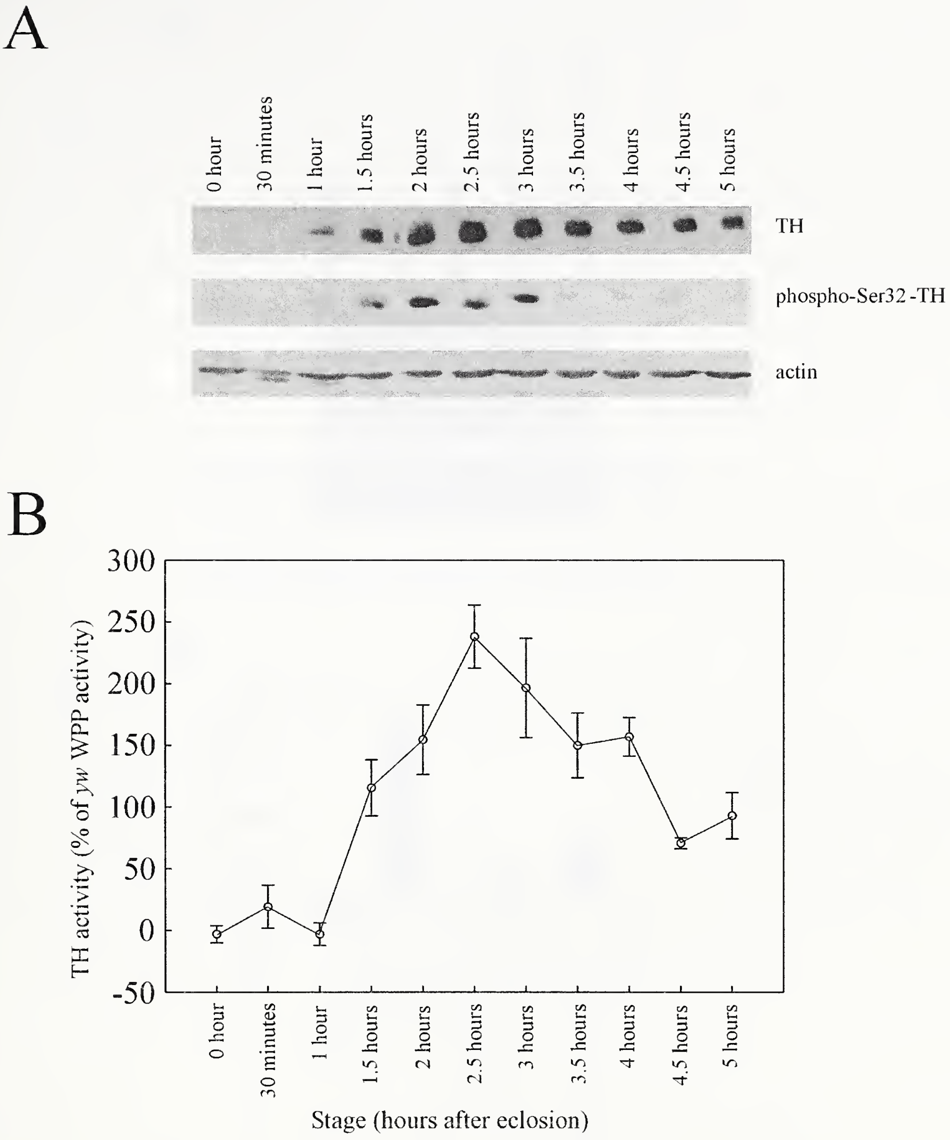

Figure 2.5: TH protein and activity in *yw* flies after eclosion. A. Western blots of epidermal TH protein and phospho-Ser32-TH. B. TH activity in the same organisms. Values are averaged over a minimum of three independent trials.

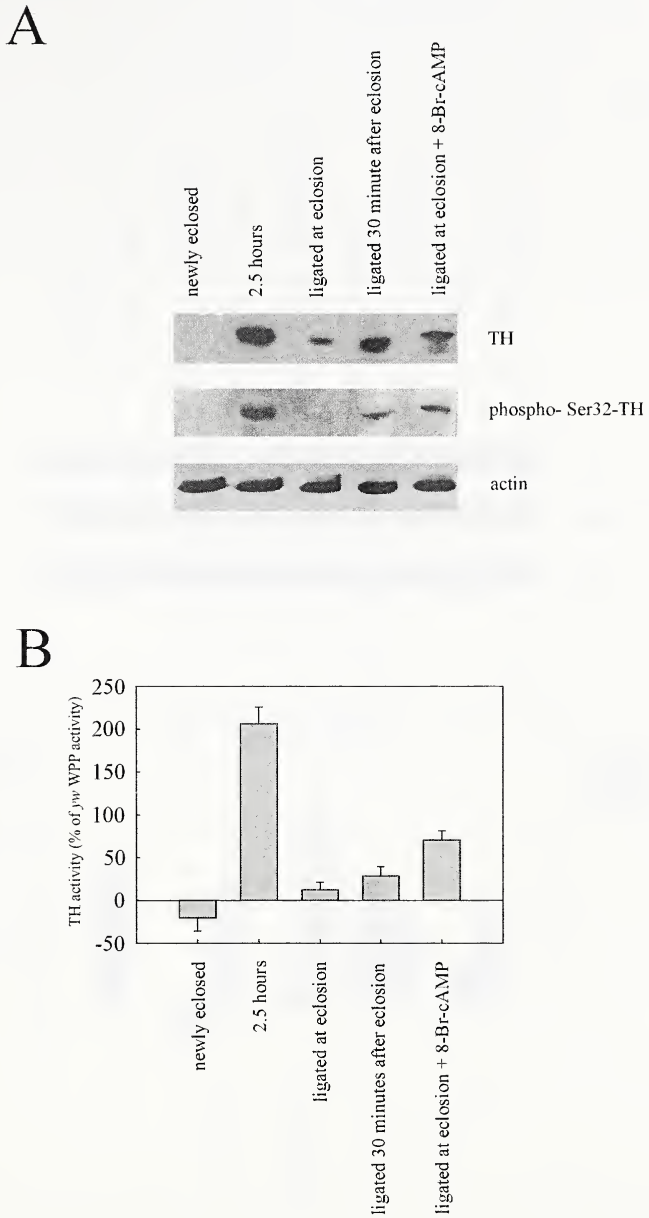

Figure 2.6: TH protein, phosphorylation and activity in neck-ligated flies. A. Western blots showing TH and phospho-Ser32-TH in *yw* flies neck-ligated at eclosion or 30 minutes thereafter and rescue by injection of 8-Br-cAMP. B. TH activity in the same organisms. Each value is an average of a minimum of three independent experiments.

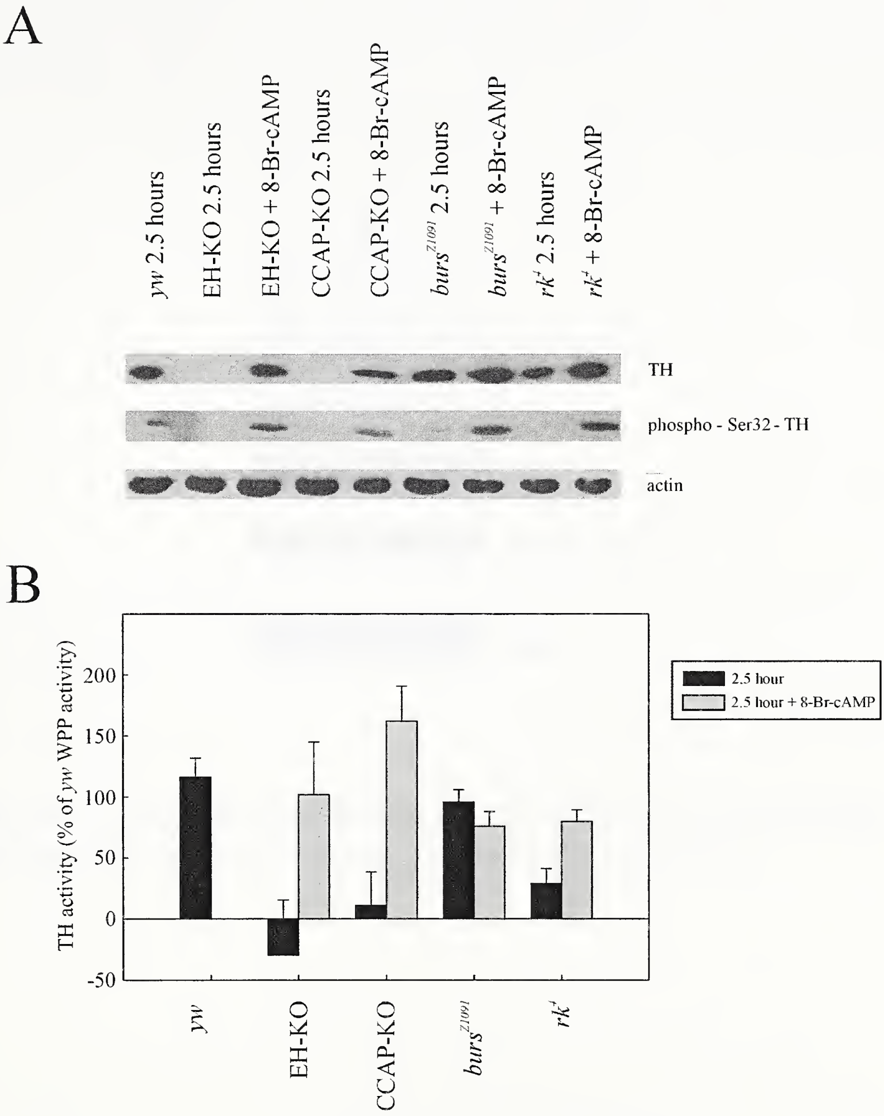

Figure 2.7: Effects of 8-Br-cAMP on TH protein accumulation, phosphorylation and activity in peptide hormone mutants. A. Western blots showing epidermal TH and phospho-Ser32-TH levels 2.5 hours after eclosion in untreated flies or flies injected with 8-Br-cAMP at eclosion. B. TH activity in the same organisms. Each value is an average of a minimum of three independent experiments.

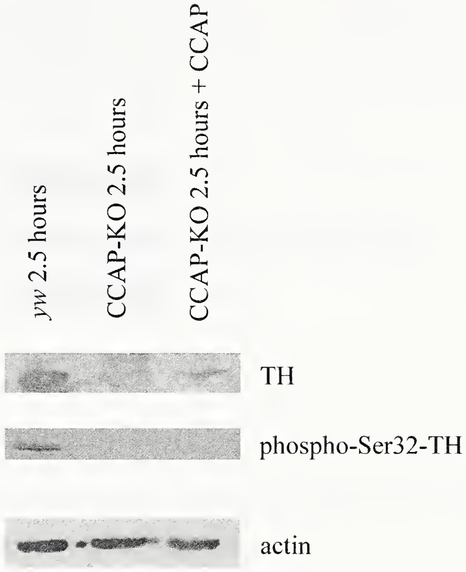

Figure 2.8: Levels of TH and phospho-Ser32-TH in 2.5 hour old *yw* and CCAP-KO control organisms and in CCAP-KO flies injected at eclosion with CCAP.

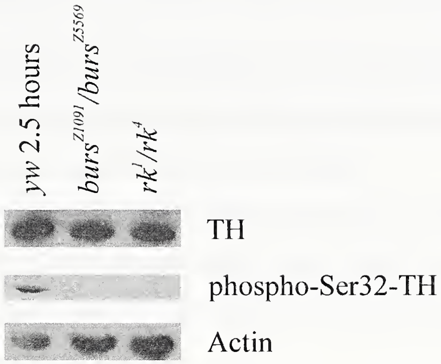

Figure 9: Levels of TH and phospho-Ser32-TH in 2.5 hour old *yw*, *burs*^{Z1091}/*burs*^{Z5569} and *rk*^{1}/*rk*^{4} flies.

References

1. Riddiford, L.M. (1993). In: Bates, M. and Arias, A.M. (eds.), The development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York, Vol. 2, pp. 899-939.
2. Curtis, A.T., Hori, M., Green, J.M., Wolfgang, W.J., et al. (1984). Ecdysteroid regulation of the onset of cuticular melanization in allatectomized and *Black* mutant *Manduca sexta* larvae. J Insect Physiol. 30: 597-599.
3. Truman, J.W. (1996). Ecdysis control sheds another layer. Science. 271: 40-41.
4. Park, Y., Filippov, V., Gill, S.S. and Adams, M.E. (2002). Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development. 129: 493-503.
5. Clark, A.C., del Campo, M.L. and Ewer, J. (2004). Neuroendocrine control of larval ecdysis behavior in *Drosophila*: complex regulation by partially redundant neuropeptides. J Neurosci. 24: 4283-4292.
6. Horodyski, F.M., Ewer, J., Riddiford, L.M. and Truman, J.W. (1993). Isolation, characterization and expression of the eclosion hormone gene of *Drosophila melanogaster*. Eur J Biochem. 215: 221-228.
7. Ewer, J., Gammie, S.C. and Truman, J.W. (1997). Control of insect ecdysis by a positive feedback endocrine system: roles of eclosion hormone and ecdysis triggering hormone. J Exp Biol. 200: 869-881.
8. Kingan, T.G. and Adams, M.E. (2000). Ecdysteroids regulate secretory competence in Inka cells. J Exp Biol. 203: 3011-3018.
9. Kingan, T.G., Cardullo, R.A. and Adams, M.E. (2001). Signal transduction in eclosion hormone-induced secretion of ecdysis-triggering hormone. J Biol Chem. 276: 25136-25142.
10. Baker, J.D., McNabb, S.L. and Truman, J.W. (1999). The hormonal coordination of behavior and physiology at adult ecdysis in *Drosophila melanogaster*. J Exp Biol. 202: 3037-3048.
11. Truman, J.W., Mumby, S.M. and Welch, S.K. (1980). Involvement of cyclic GMP in the release of stereotyped behavior patterns in moths by a peptide hormone. J Exp Biol. 84: 201-212.

12. Gammie, S.C. and Truman, J.W. (1999). Eclosion hormone provides a link between ecdysis-triggering hormone and crustacean cardioactive peptide in the neuroendocrine cascade that controls ecdysis behavior. J Exp Biol. 202: 343-352.
13. Ewer, J. and Truman, J.W. (1996). Increases in cyclic 3',5'-guanosine monophosphate (cGMP) occur at ecdysis in an evolutionarily conserved crustacean cardioactive peptide-immunoreactive insect neuronal network. J Comp Neurol. 370: 330-341.
14. Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., et al. (2003). Targeted ablation of CCAP neuropeptide-containing neurons of *Drosophila* causes specific defects in execution and circadian timing of ecdysis behavior. Development. 130: 2645-2656.
15. Gammie, S.C. and Truman, J.W. (1997). Neuropeptide hierarchies and the activation of sequential motor behaviors in the hawkmoth, *Manduca sexta*. J Neurosci. 17: 4389-4397.
16. Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., et al. (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in *Drosophila*. J Neurosci. 26: 573-584.
17. Dewey, E.M., McNabb, S.L., Ewer, J., Kuo, G.R., et al. (2004). Identification of the gene encoding *bursicon*, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr Biol. 14: 1208-1213.
18. Luo, C.W., Dewey, E.M., Sudo, S., Ewer, J., et al. (2005). Bursicon, the insect cuticle-hardening hormone, is a heterodimeric cystine knot protein that activates G protein-coupled receptor LGR2. Proc Natl Acad Sci USA. 102: 2820-2825.
19. Mendive, F.M., Van Loy, T., Claeysen, S., Poels, J., et al. (2005). Drosophila molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. Febs Lett. 579: 2171-2176.
20. Baker, J.D. and Truman, J.W. (2002). Mutations in the *Drosophila* glycoprotein hormone receptor, *rickets*, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J Exp Biol. 205: 2555-2565.

21. Kimura, K., Kodama, A., Hayasaka, Y. and Ohta, T. (2004). Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*. Development. 131: 1597-1606.
22. Fraenkel, G. and Hsiao, C. (1962). Hormonal and nervous control of tanning in fly. Science. 138: 27-29.
23. Fraenkel, G., Hsiao, C. and Seligman, M. (1966). Properties of bursicon: an insect protein hormone that controls cuticular tanning. Science. 151: 91-93.
24. McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., et al. (1997). Disruption of a behavioral sequence by targeted death of peptidergic neurons in *Drosophila*. Neuron. 19: 813-823.
25. Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., et al. (2003). EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. Development. 130: 271-284.
26. Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet. 24: 127-222.
27. Nagatsu, T., Levitt, M. and Udenfriend, S. (1964). Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem. 239: 2910-2917.
28. Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965). Elucidation of rate-limiting step in norepinephrine biosynthesis in perfused guinea-pig heart. J Pharmacol Exp Ther. 148: 1-8.
29. Neckameyer, W.S. and White, K. (1993). *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. J Neurogenet. 8: 189-199.
30. Livingstone, M.S. and Tempel, B.L. (1983). Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. Nature. 303: 67-70.
31. Hirsh, J. and Davidson, N. (1981). Isolation and characterization of the *Dopa decarboxylase* gene of *Drosophila melanogaster*. Mol Cell Biol. 1: 475-485.
32. Scholnick, S.B., Morgan, B.A. and Hirsh, J. (1983). The cloned *Dopa decarboxylase* gene Is developmentally regulated when reintegrated into the *Drosophila* genome. Cell. 34: 37-45.

33. Birman, S., Morgan, B., Anzivino, M. and Hirsh, J. (1994). A novel and major isoform of tyrosine hydroxylase in *Drosophila* is generated by alternative RNA processing. J Biol Chem. 269: 26559-26567.
34. Morgan, B.A., Johnson, W.A. and Hirsh, J. (1986). Regulated splicing produces different forms of *Dopa decarboxylase* in the central nervous system and hypoderm of *Drosophila melanogaster*. EMBO J. 5: 3335-3342.
35. Wright, T.R., Bewley, G.C. and Sherald, A.F. (1976). The genetics of dopa decarboxylase in *Drosophila melanogaster*. II. Isolation and characterization of dopa-decarboxylase-deficient mutants and their relationship to the alpha-methyl-dopa-hypersensitive mutants. Genetics. 84: 287-310.
36. Friggi-Grelin, F., Iche, M. and Birman, S. (2003). Tissue-specific developmental requirements of *Drosophila* tyrosine hydroxylase isoforms. Genesis. 35: 260-269.
37. Zigmond, R.E., Schwarzschild, M.A. and Rittenhouse, A.R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annu Rev Neurosci. 12: 415-461.
38. Hufton, S.E., Jennings, I.G. and Cotton, R.G.H. (1995). Structure and function of the aromatic amino acid hydroxylases. Biochem J. 311: 353-366.
39. Kumer, S.C. and Vrana, K.E. (1996). Intricate regulation of tyrosine hydroxylase activity and gene expression. J Neurochem. 67: 443-462.
40. Dunkley, P.R., Bobrovskaya, L., Graham, M.E., von Nagy-Felsobuki, E.I., et al. (2004). Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem. 91: 1025-1043.
41. Vie, A., Cigna, M., Toci, R. and Birman, S. (1999). Differential regulation of *Drosophila* tyrosine hydroxylase isoforms by dopamine binding and cAMP-dependent phosphorylation. J Biol Chem. 274: 16788-16795.
42. Kraminsky, G.P., Clark, W.C., Estelle, M.A., Gietz, R.D., et al. (1980). Induction of translatable mRNA for *Dopa decarboxylase* in *Drosophila*: an early response to ecdysterone. Proc Natl Acad Sci USA. 77: 4175-4179.
43. McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., et al. (1997). Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila. Neuron. 19: 813-823.

44. Lunan, K.D. and Mitchell, H.K. (1969). Metabolism of tyrosine-*O*-phosphate in *Drosophila*. Arch Biochem Biophys. 132: 450-456.

45. Bel, Y., Jacobson, K.B., Silva, F.J. and Ferre, J. (1992). Developmental and biochemical studies on the phenylalanine hydroxylation system in *Drosophila melanogaster*. Insect Biochem Mol Biol. 22: 633-638.

46. Denman, R.B., Dolzhanskaya, N. and Sung, Y.J. (2004). Regulating a translational regulator: mechanisms cells use to control the activity of the fragile X mental retardation protein. Cell Mol Life Sci. 61: 1714-1728.

47. Wilhelm, J.E. and Smibert, C.A. (2005). Mechanisms of translational regulation in *Drosophila*. Biol Cell. 97: 235-252.

48. Enright, A.J., John, B., Gaul, U., Tuschl, T., et al. (2003). MicroRNA targets in *Drosophila*. Genome Biol. 5: R1.

Chapter 3: A member of the p38 MAPK family is responsible for inducing transcription of *Dopa decarboxylase* in the epidermis of *Drosophila melanogaster* during the innate immune response

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Introduction

Most animal species exhibit the ability to ward off infection. While mammals employ a combination of adaptive and innate immune responses, the majority of organisms use only innate immunity to combat infection. The genetic and molecular techniques available in *Drosophila melanogaster* have made it an exceptional model for innate immune research (1). Drosophila immunity combines humoral and cellular responses to effect a strong resistance to many microorganisms. The three mechanisms contributing to this resistance are: phagocytosis of invading organisms by haemocytes; blood clotting, melanin formation and opsonisation; and transient synthesis of antimicrobial peptides (AMPs) at the wound site and in the fat body.

Drosophila lives in decaying and fermenting matter, and therefore is exposed to a multitude of bacteria, fungi, and viruses that it must defend itself against. The ability to combat natural and septic infections by gram negative and gram positive bacteria has been the focus of much study in the last several years. The work has centered on the humoral defence response, involving the synthesis and release of AMPs from the fat body into the haemolymph. Transcription of different but overlapping sets of approximately 20 AMPs (2,3) is elicited by gram negative bacteria upon activation of the IMD pathway (4-6) and gram positive bacteria and fungi following induction of the Toll pathway (7,8). Each pathway acts through its respective NFκB transcription factor(s), the Toll pathway uses Dorsal and Dif (9-11), while the IMD pathway activates Relish (12).

Immune activation of the IMD pathway also causes the induction of the mitogen activated protein kinase (MAPK) cascade, known as the Jun kinase (JNK) pathway, due to a bifurcation at the TGF-Activated kinase, TAK1 (13-15). In Drosophila, the MAPK kinase (MAPKK), Hemipterous (HEP) (16) activates the JNK, Basket (BSK) (13), that can then phosphorylate the heterodimeric transcription factor AP-1 (17,18), which is composed of a Fos and a Jun subunit (19,20). Phosphorylation activates AP-1 and causes

induction of JNK response genes. JNK signalling is required for proper wound healing in the adult epidermis (21,22) and the wing disc (23). JNK signalling also activates the transcription of many proteins involved in cytoskeleton remodelling (13), consistent with its role in haemocyte activation in the cellular immune response (24). A role for JNK signalling in AMP gene expression has been proposed (25,26); however, further research needs to be done because JNK repression of AMP synthesis has also been proposed (27).

In addition to the JNK pathway, two other conserved MAPK pathways potentially have roles in Drosophila immunity and wound healing. The first, the extracellular signal-regulated kinase (ERK) pathway, has been implicated in the Drosophila embryonic wound response (28). The second is the p38 MAPK family, which has two characterized members (p38a and p38b) that have been implicated in attenuation of the immune response (29). p38 MAPKs are activated by dual phosphorylation by a MAPKK at a characteristic TGY site (30). This site differs from the dual phosphorylation TPY and TEY motifs of JNK and ERK, respectively.

The primary layer of defence against infection involves the barrier epithelia of the epidermis, including the hypoderm that lies beneath the cuticle, and the epithelial tissues of the trachea and gut. The barrier epithelia are responsible for localized production of AMPs at the site of septic injury (31,32). While formation of a melanin clot at the wound site is not specifically an immune response, it is essential to prevent haemolymph loss following septic injury. An additional benefit is that the rapid production of the melanised clot entraps bacteria, and promotes killing. The melanin produced at the clot site is a direct consequence of the catalytic activation of the zymogen prophenol-oxidase (33), that is stored in the crystal cells (34,35), to produce active phenol oxidase (PO). PO uses tyrosine to produce the quinones that react non-enzymatically to form melanin (35,36). Clots form without the action of PO (33,37); however, PO is required for clot hardening (33,38). An additional potential role for melanin production at the wound site involves the formation of reactive oxygen species and byproducts of melanin production that have cytotoxic antimicrobial properties (39). Interestingly, the JNK cascade is necessary for crystal cell rupture following injury, which leads to melanisation at the wound site (40).

In addition to the role of PO in wound healing, two other genes, *pale* (*ple*) and *Dopa decarboxylase* (*Ddc*), (41), have been shown to be essential to wound healing in the Drosophila embryo (28). These genes encode the enzymes tyrosine hydroxylase (TH), which converts tyrosine to dihydroxyphenylalanine (Dopa), and Dopa decarboxylase (DDC), which converts Dopa to dopamine (DA). The neural role of DA is well established; however, DA is also metabolized to produce melanin and sclerotin in epidermal tissues (41,42), which leads to tanning of the pupal case and adult cuticle, and pigmentation of the pharate adult epidermis (43). The transcription factor Grainy-head is reported to be responsible for *Ddc* induction in the embryonic wound response, by acting through a binding site just upstream of the transcription start site (28). However, deletion of 3 AP-1 sites and a CREB (an ERK inducible transcription factor) binding site upstream of *Ddc* also eliminates expression of *Ddc* during the wound response, indicating a potential role for the JNK or ERK pathways in this induction. Consistent with the role of a MAPK in *Ddc* transcriptional induction, injection of the MAPK inhibitor PD98059 into embryos reduced the induction of a *Ddc*-GFP reporter after aseptic injury.

The potential role for *Ddc* in the Drosophila innate immune response in larvae and adults is interesting. Defence against the parasitic wasp *Leptopilina boulardii* requires the production of a melanised capsule that envelops the egg, and eventually kills it. Melanotic encapsulation requires the actions of specialized blood cells called lamellocytes (35,36). Nappi et al. (44) showed that the melanisation response was severely compromised in *Ddc*^{ts2} flies, demonstrating the need for functional DDC in the encapsulation response. *Ddc* transcription was induced in larvae of *Tenebrio molitor* infected with *Escherichia coli* (45), and this corresponded to increased DDC protein and activity levels. In addition, N-β-alanine (NBAD) synthase, an enzyme that converts DA to the sclerotin precursor, NBAD, is induced in the epidermis of *T. molitor* and *Ceratitis capitata* following *E. coli* infection (46). NBAD has potent antimicrobial effects, which can be removed by addition of antioxidants, suggesting that NBAD elicits its killing effects due to spontaneous conversion to cytotoxic quinones. Together, these results suggest a role for DA metabolism in the insect innate immune response. Consistent with this prediction, microarray experiments have identified *Ddc* as one of the genes induced

after both gram positive and gram negative bacterial infection, but not fungal infection of Drosophila (47-49).

In this paper, we investigate the role of *Ddc* in the Drosophila innate immune response to bacterial infection. We find that *Ddc* is induced upon septic injury, but not aseptic wounding of larvae and adults, and this response is dependent on a single conserved AP-1 binding site upstream of the *Ddc* transcription start site. However, the JNK pathway does not induce transcription of *Ddc*; rather, an uncharacterized p38 MAPK, p38c, is responsible. Induction of *Ddc* occurs throughout the epidermis and is not restricted to the wound site. We propose that DDC activity is required in the epidermis for the synthesis of DA that is metabolized to produce reactive quinones that exert killing effects on invading bacteria.

Results

Ddc transcription is induced in the Drosophila innate immune response

Microarray studies of Drosophila immunity have identified *Ddc* as an immune inducible gene (47-49). To confirm that *Ddc* transcription is induced by bacterial infection, we performed semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) on uninjured, aseptically injured, and septically injured mid-third instar larvae and adult flies, harvested 4 hours after treatment (Figure 3.1). The *Ddc* forward and reverse PCR primers are anchored in the first and third exons of *Ddc*, respectively, and the PCR conditions are such that we never amplify the larger product that contains the neural-specific 2nd exon (Figure 3.2). Transcription of *Ddc* is induced when larvae or adults are infected with *E. coli* (gram negative) (Figure 3.1, lane 4) or *Staphylococcus aureus* (gram positive) (lane 5) and when organisms are aseptically injured with a large needle (data not shown); however, little *Ddc* transcript is detectible when organisms are aseptically injured with a small needle (lane 3) or left untreated (lane 2). We find that if organisms are septically injured with a small needle, *Ddc* transcription is induced, whereas if that needle is used for aseptic injury, *Ddc* transcription is absent. We attribute *Ddc* induction following aseptic injury with a large needle to infection with natural flora on the Drosophila cuticle.

Ddc transcription is localized to the epidermis

Having determined that *Ddc* is induced in the immune response, we next set out to identify which tissues express *Ddc*. To do this, we aseptically and septically injured adult flies and 4 hours later separated their heads, thoraxes, and abdomens for RNA isolations. We find that *Ddc* transcription is induced strongly in the head and thorax, and less so in the abdomen of the flies that were septically injured with either gram positive or gram negative bacteria (Figure 3.3A). This induction was absent in the flies that were uninjured or aseptically injured controls. This suggests that *Ddc* transcription is induced throughout the organism, and not restricted to the site of infection.

Since *Ddc* transcription is induced throughout the adult organism, the three most likely locations of transcriptional induction are in the haemocytes, the epidermis, or the fat body. In order to determine if *Ddc* transcription is induced in the haemolymph, we examined induction of *Ddc* transcription in larvae of *domino* (*dom*) mutants that fail to develop functional haemocytes (50). We were unable to assess *Ddc* induction in adult dom^{K08108} flies, as these mutants die at pupariation when large numbers of haemocytes are necessary for phagocytosis at the onset of metamorphosis. However, we find that *Ddc* transcription is induced normally in dom^{K08108} larvae (Figure 3.3B, lanes 3 and 4), and that these mutants appear to have a high basal level of *Ddc* transcription (lane 2), likely due to chronic infection caused by the lack of haemocyte derived cellular defences.

To distinguish between the epidermis and the fat body as the site of *Ddc* transcriptional induction, these tissues were isolated by dissection from mid-third instar larvae four hours after septic injury. We isolated tissues from larvae rather than adults, since the fat body tissues are distributed throughout the adult fly (51) and epidermal tissues are difficult to dissociate from cuticular tissues in Drosophila adults. Semi-quantitative RT-PCR on the RNA obtained from these samples reveals that *Ddc* transcription is induced in the epidermal tissues and not the fat body (Figure 3.3C).

Reduction of DDC activity has no effect on adult survival following infection

To determine if DDC activity within the epidermis is required for survival in the Drosophila immune response, we infected Ddc^{ts2}/Ddc^{n7} and $Ddc^{ts2}/Df(2L)130$ mutants with *E. coli* and *S. aureus*, and tracked their survival for 3 days (Table 3.2). The Ddc^{n7} allele is a null mutation and *Df(2L)130* is a deficiency of the locus; both are embryonic

lethal. The *Ddc*^{ts2} mutation is a temperature sensitive mutation, and *Ddc*^{ts2}/*Df(2L)130* flies have 2-3% of the DDC activity detectible in wild-type flies (52). We find that there is no difference in the survival of *Ddc*^{ts2}/*Ddc*^{n7} (Table 3.2A) and *Ddc*^{ts2}/*Df(2L)130* (Table 3.2B) compared to their wild-type siblings. Thus, we conclude that *Ddc* induction at 2-3 % of normal has no affect on 3 day adult survival following infection with *E. coli* and *S. aureus*.

In an attempt to eliminate DDC activity completely, we employed RNAi to degrade *Ddc* transcripts. To confirm that our *Ddc*-RNAi constructs were functional, we expressed the UAS driven RNAi constructs under the control of an *Act5C*-Gal4 driver. We found that all of the *Act5C*-Gal4; P[*Ddc*-RNAi]pWIZ flies died at pupariation, a stage when a high level of DDC activity is required for the rapid tanning of the pupal case. However, it is unlikely that there is a complete loss of DDC activity in these flies, as *Ddc* null mutants are embryonic lethal (53). *Act5C*-Gal4; P[*Ddc*-RNAi]pSymp flies developed normally, suggesting that expression of this construct did not destroy all of the *Ddc* transcripts, so we eliminated these flies from further analysis. We infected flies of the genotype *hs*-Gal4; P[*Ddc*-RNAi]pWIZ with or without heat shock and found that there was no difference in lethality between heat shock and control flies following infection (Table 3.2C). Thus, it appears that reduction in DDC activity in these flies does not affect adult survival.

The time course of *Ddc* transcription is different in gram negative and gram positive infections

While reducing DDC activity did not decrease the survival of infected flies, our results do show that *Ddc* transcription is induced in the immune response. We set out to determine the transcriptional profile of *Ddc* expression following infection with *E. coli* or *S. aureus* (Figure 3.4). Adult flies were septically injured with live or heat-killed bacteria and harvested at various time points. Since *Ddc* transcript levels are high at the 2-3 moult and at pupariation during normal development (M. M. Davis, unpublished), the time course of *Ddc* transcription following septic injury was not determined for third instar larvae. RT-PCR on RNA isolated from pools of adults collected at the indicated time points reveals that *Ddc* transcripts are first detectible about 1 hour after infection with live or heat-killed *E. coli* (Figure 3.4, top gel). Levels peak within 3 hours, remain high

until 6 hours after infection and drop thereafter. The profile of *Ddc* transcription is different in response to septic injury with live *S. aureus* (Figure 3.4, bottom gel). In this case, *Ddc* transcripts are first detected 2 hours after infection; levels peak by 9 hours and drop slowly thereafter. Similar to infection with live *S. aureus*, flies septically injured with heat-killed *S. aureus* induce *Ddc* transcription, and transcripts are detectible 3 and 6 hours after infection. However, *Ddc* transcripts are not detectible 9 and 12 hours after infection with heat-killed *S. aureus*. Thus, the sustained response of *S. aureus* induction of *Ddc* transcription requires continued infection by live bacteria.

A conserved AP-1 binding site is necessary for the induction of *Ddc* transcription

To determine which regulatory sequence was responsible for *Ddc* induction in the immune response, we created *Ddc*-GFP reporter constructs containing varying amounts of the *Ddc* 5'regulatory region (Figure 3.2). Epidermal autofluorescence made confocal images nearly impossible to analyze, so we employed semi-quantitative RT-PCR to examine the expression of the reporter constructs. First, we infected flies bearing the constructs that contain the large deletions, P[*Ddc*-GFP]SH, P[*Ddc*-GFP]BH, and P[*Ddc*-GFP]EH, and compared reporter gene induction in these flies to expression in P[*Ddc*-GFP]PH bearing flies (Figure 3.5A, top gel). For all *Ddc*-GFP constructs, we examined expression following infection in a minimum of 3 independent transgenic lines. We find that the reporter gene is induced normally following both gram negative and gram positive infection in flies bearing the P[*Ddc*-GFP]PH, P[*Ddc*-GFP]SH, and P[*Ddc*-GFP]BH constructs. However, GFP transcription is not induced in flies bearing the P[*Ddc*-GFP]EH construct. This suggests that the sequence necessary for *Ddc* induction is located between the *Bsm*I and *Eco*RI restriction sites (Figure 3.2). To confirm this, we infected a transgenic line bearing a construct, P[*Ddc*-GFP]PHΔBE, that deleted only this region (Figure 3.2), and found that the reporter gene could not be induced following septic injury with *E. coli* or *S. aureus*. For each construct-bearing line, the wild-type copy of the endogenous *Ddc* gene was induced (Figure 3.5A, bottom gel), showing that the immune response was triggered normally. To further narrow down the region responsible for *Ddc* induction, we created transgenic flies bearing reporter constructs that contained a deletion of part of the region between the *Bsm*I and *Eco*RI sites. These were called P[*Ddc*-GFP]ΔBE-1, P[*Ddc*-GFP]ΔBE-2, and P[*Ddc*-GFP]ΔBE-3 (Figure 3.2). We

find that reporter gene transcription is immune inducible in flies bearing P[*Ddc*-GFP]ΔBE-1 and P[*Ddc*-GFP]ΔBE-2, but not in flies carrying P[*Ddc*-GFP]PHΔBE-3 (Figure 3.5B, top gel). We conclude that a sequence contained within this 302 bp region is necessary for the induction of *Ddc* transcription in the innate immune response.

We aligned this 302 bp region (using the BLAT program; http://genome.ucsc.edu/) (54) with nine other sequenced Drosophila genomes. The sequence was also scanned for the presence of binding sites for known transcription factors found in a database (TESS) (55). This analysis revealed two conserved binding sites that could be involved in the Drosophila innate immune response (Figure 3.6). These sites include the 7 bp AP-1 binding site (bold and underlined) and the 8 bp NFκB binding site (bold and capital letters).

To determine if either of these sites were necessary for *Ddc* transcriptional induction in the innate immune response, we used site directed mutagenesis (See Appendix 2) to mutate 5 bases within each site to create P[*Ddc*-GFP]PHmutNFκB and P[*Ddc*-GFP]PHmutAP-1 (Figure 3.2). When transgenic flies bearing P[*Ddc*-GFP]PHmutNFκB were infected with either *E. coli* or *S. aureus*, reporter gene transcription was induced (Figure 3.5B, top gel). This suggests that the consensus NFκB site plays no role in *Ddc* immune induction. The importance of an intact consensus AP-1 site is apparent from the lack of reporter gene transcription in P[*Ddc*-GFP]PHmutAP-1 flies following infection with either bacterium. In both cases, septic injury induced transcription of the endogenous *Ddc* normally in reporter bearing flies (Figure 3.5B, bottom gel). An intact consensus AP-1 site was also necessary for larval transcriptional induction (data not shown).

AP-1 can bind its putative target site *in vitro*

Loss of reporter gene induction in P[*Ddc*-GFP]PHmutAP-1suggests that the JNK activated transcription factor complex, AP-1, may be involved in the activation of *Ddc* transcription in the innate immune response. To determine if AP-1 could bind its putative binding site upstream of *Ddc*, we employed an electrophoretic mobility shift assay (Figure 3.7). We created a 26 bp probe containing the conserved *Ddc* AP-1 sequence, and a probe with changes in the same 5 bases that were mutated in P[*Ddc*-GFP]PHmutAP-1. We find that when we add reticulocyte lysate mix alone, to either the

wild-type or mutant probe, we see retardation of the probe (lane 2 vs. lane 1 and lane 10 vs. lane 9). We do not see an additional shift of the wild-type probe when we add Jun or Fos alone (lanes 3 and 4 respectively). However, if we add Jun and Fos together, producing a complete AP-1 transcription factor, we see further retardation of the probe (arrow, lanes 5 and 6). This product is lost when either 100 times or 200 times cold competitor DNA is added to the reaction (lanes 7 and 8 respectively). However, addition of both Jun and Fos does not cause further retardation of the mutant probe (lanes 11 and 12), suggesting that AP-1 is unable to bind to the mutated binding site.

Neither the Toll, IMD, nor JNK signalling is involved in *Ddc* immune induction

To determine if the consensus AP-1 binding site was a target of JNK signalling, we employed the bipartite Gal4/UAS system to express wild-type and dominant negative forms of the Drosophila MAPKK, HEP, and the JNK, BSK, as well as Jun and Fos under the control of a heat shock promoter. In each case, we infected organisms that had been heat shocked for 1 hour and then allowed to recover for 1 hour prior to infection. Control organisms were not heat shocked. Expression of *hep* (UAS-*hep*), *bsk* (UAS-*bsk*), *fos* (UAS-*fos*), or *jun* (UAS-*jun*) did not precociously induce *Ddc* transcription in the absence of infection, although *Ddc* was immune inducible in these flies (Figure 3.8). Furthermore, expression of a constitutively active HEP (UAS-*hepACT*) did not precociously induce *Ddc* transcription (Figure 3.8A). Finally, expression of dominant negative forms of *bsk* (UAS-*bskDN*), fos (UAS-*fosDN*), or *jun* (UAS-*junDN*) did not eliminate immune induction of *Ddc* transcription (Figure 3.8B,C,D).

Ectopic expression of these JNK pathway components failed to indicate a role for JNK signalling in the *Ddc* immune response. Consistent with this observation, *Ddc* was not shown to be a JNK inducible gene (47). However, since a conserved AP-1 binding site is necessary for *Ddc* immune induction, we thought it advisable to assess the effects of a JNK loss of function mutant on *Ddc* inducibility. *Tak1* mutant flies, in which the mutation eliminates both IMD and JNK signalling (14,15), activate immune transcription of *Ddc* normally (Figure 3.9A). This provides strong evidence that the JNK pathway, and its transcription factor complex, AP-1, are not involved in the induction of *Ddc* in the innate immune response.

We also infected mutants of additional components of the IMD pathway. These included *ird5*^{KG08072} and *ird5*^{EY02434}, both of which have a P-element insertion in the first exon of the *ird5* gene, which encodes the β subunit of the IKK complex (56-58). This complex is essential for IMD signalling downstream of TAK1. In addition, we infected flies bearing a P-element insertion in the gene encoding the IMD-induced NFκB transcription factor, Relish, *Rel*^{EY08061}. *Ddc* transcription was immune inducible in these each of these mutants (data not shown). Genetic data likewise fail to indicate a role for Toll signalling in *Ddc* immune induction. The *Tl*^{3} gain-of-function mutant, which constitutively induces AMP expression in the absence of infection (7), did not precociously induce *Ddc* transcription (data not shown). This provides further evidence that the NFκB site, although perfectly conserved, is not required for *Ddc* induction following septic injury.

The p38c MAPK pathway is responsible for *Ddc* induction

We have provided evidence that the major immune signalling pathways are dispensable for *Ddc* immune induction. Two other MAPK cascades have been implicated in Drosophila immunity and wound healing. The first, the ERK pathway, was shown by Mace et al. (2005) to be necessary for robust induction of *Ddc* in the embryonic wound response. The Drosophila ERK is encoded by the gene *rolled* (*rl*) (59). We infected *rl*^{1} mutants with *E. coli* and *S. aureus*, and found that *Ddc* was induced normally in these mutants (Figure 3.9B).

The second, the p38 MAPK pathway, has been implicated in the attenuation of the immune response (29). There are two p38 MAPKs that have been described in Drosophila, *p38a* and *p38b*. In addition, there is a third p38, termed *p38c* (CG33338), which has been annotated in FlyBase. The *p38c* gene is located immediately downstream of *p38a*, and likely arose by gene duplication (60). Although *p38c* shares 61% DNA sequence identity to *p38a*, there is some doubt that it, in fact, encodes a functional p38 MAPK. The *p38c* gene has a mutation that changes the TGY domain that is characteristic of p38 MAPKs (30) to a TDH (61), thereby preventing this motif from serving as a substrate for dual phosphorylation. Secondly, it has two amino acid substitutions within the eight amino acid catalytic domain that could prevent the active site from functioning. However, 4 expressed sequence tags (ESTs) were identified in a

cDNA library obtained from the larval fat body of infected organisms (62). Furthermore, the p38c MAPK protein coding sequence is conserved, suggesting that *p38c* is not a pseudogene, and may, in fact, be functional (60).

The *Mpk2*^{1} null allele of *p38a* (63), along with P-element insertions upstream of *p38b* (*p38b*^{KG01337} and *p38b*^{KG02737}) and within *p38c* (*p38c*^{KG05834}) were obtained and infected with *E. coli* or *S. aureus* (Figure 3.10A). We find that *Ddc* transcription is induced normally in *Mpk2*^{1}, *p38b*^{KG01337} (Figure 3.10A) and *p38b*^{KG02737} (data not shown) flies, but is not induced in *p38c*^{KG05834} flies (Figure 3.10A). The *p38c* ^{KG05834} allele contains an insertion of a P-element, in the center of the coding sequence, which most likely disrupts p38c function. However, this insertion is annotated in FlyBase as affecting *p38a* function. We performed RT-PCR for both *p38a* and *p38c* transcripts on RNA obtained from *Mpk2*^{1} and *p38c* ^{KG05834} mutants to determine the nature of the *p38c*^{KG05834} mutation. We find that *p38a* is transcribed in *p38c*^{KG05834} flies but not *Mpk2*^{1} flies (Figure 3.10B), and *p38c* transcript is detectible in *Mpk2*^{1} mutants, but not in *p38c*^{KG05834} flies (Figure 3.10C). Thus, we conclude that the insertion in *p38c*^{KG05834} affects the expression of *p38c* and not *p38a* in these flies.

In an attempt to analyse *p38c* expression, we used quantitative RT-PCR to detect *p38c* transcripts in uninjured and septically injured adult flies (Figure 3.10D). We find that *p38c* transcription is not immune inducible; rather, levels of *p38c* transcripts are similar in infected and uninfected organisms.

Discussion

Microarray analysis has shown that *Ddc* transcription is induced by infection with gram negative and gram positive bacteria (47-49). We have confirmed that *Ddc* (Figure 3.1), but not *ple* (data not shown) transcription is induced following gram negative (*E. coli*) or gram positive (*S. aureus*) septic injury with a small needle. The lack of *ple* transcriptional induction is not surprising, since PO activity can provide Dopa for DDC, thereby making TH redundant. In addition, transcripts for the genes encoding Dihydropteridine reductase and GTP cyclohydrolase I, which are responsible for production of the essential TH co-factor tetrahydrobiopterin, are significantly upregulated following septic injury (47-49). This induction is likely sufficient to increase the basal TH activity levels.

Aseptic wounding of larvae or adults does not lead to *Ddc* induction (Figure 3.2), unlike in embryos where *Ddc* induction at the edge of the wound contributes to the formation of a melanin clot (28). Our data show that robust induction of *Ddc* transcription is not apparent until 2 hours after infection (Figure 3.4), long after the melanin clot has already formed at the wound site. The profile of *Ddc* transcription differs in response to live *E. coli* and *S. aureus* infection (Figure 3.4A). This discrepancy can be attributed to the virulence of *S. aureus*, since infection with heat-killed *S. aureus* recapitulates the profile of *Ddc* transcriptional induction with live *E. coli* (Figure 3.4B).

Induction of *Ddc* transcription following septic injury (Figure 3.3A) occurs throughout the epidermis and not in the fat body (Figure 3.3C). Although we do not see a change in infection induced *Ddc* transcription in dom^{K08108} larvae (Figure 3.3B), we cannot eliminate the possibility that the haemocytes also express *Ddc*. Induction of *Ddc* transcription in the epidermal tissues following infection (Figure 3.3C), but not wounding (Figure 3.2) suggests a role for *Ddc* in the destruction of invading microorganisms. We find that mutations in *Ddc*, or knock-down of *Ddc* expression by RNAi does not affect the ability of Drosophila to survive infection (Table 3.2). This suggests that the low level of DDC activity in these flies is sufficient for survival. However, the actions of enzymes such as phenol oxidase in the epidermis may be able to compensate for loss of DDC activity in the immune response. Since all null alleles of *Ddc* are embryonic lethal (53), it is impossible to determine whether DDC activity is essential for survival following infection.

In an effort to determine which *Ddc* promoter element is responsible for transcriptional induction during the innate immune response, we infected larvae and adults of fly stocks bearing various *Ddc*-GFP reporter constructs (Figure 3.2, 3.5). We confirmed that the immune response was mounted in these flies by showing that transcription from the endogenous *Ddc* gene was induced (Figure 3.5A, bottom gels). Infection of flies bearing constructs revealed that a 302 bp sequence that was deleted in P[*Ddc*-GFP]PHΔBE-3 was necessary for *Ddc* induction (Figure 3.5B). Alignment of the sequences from 10 Drosophila species for this 302 bp region (Figure 3.6) revealed conserved binding sites for NFκB (capital letters and bold) and AP-1 (underlined and bold) transcription factors. Although the NFκB site is highly conserved in the 10

Drosophila species (Figure 3.6), this site is dispensable for immune induction of *Ddc* transcription (Figure 3.5B).

The Toll and IMD pathways both activate the immune response through NFκB transcription factors. Mutations in Toll (Tl^{3}) and IMD ($ird5^{KG08072}$, $ird5^{EY02434}$, and $Rel^{EY08061}$) pathway components had no effect on *Ddc* immune induction following *E. coli* or *S. aureus* infection (data not shown). This confirms that the NFκB site is not necessary for *Ddc* immune induction in larvae and adults. However, *Ddc* could be developmentally regulated by Toll signalling, particularly during embryogenesis when Toll signalling is very important for embryonic patterning (64) and the role of *Ddc* is not well understood.

Mutation of the consensus AP-1 site eliminated the immune induction of the *Ddc*-GFP reporter construct (Figure 3.5B), suggesting that a transcription factor must bind this site to induce *Ddc* transcription. Drosophila AP-1 is a heterodimer composed of one Jun and one Fos subunit (65). We demonstrated that AP-1 could bind its putative site within the *Ddc* promoter (Figure 3.7, lanes 5 and 6), but not a mutated site (lanes 11 and 12), and that binding could be eliminated by the addition of a specific competitor (lanes 7 and 8). AP-1 is activated by phosphorylation carried out by JNK (66). Using a *hs*-Gal4 driver, we induced ectopic expression of two kinases in the JNK pathway, HEP and BSK. Neither these nor a constitutively active form of HEP (UAS-*hepACT*) caused precocious induction of *Ddc* transcription in the absence of infection (Figure 3.8A,B). In addition, induction of dominant negative forms of HEP and BSK did not prevent induction of *Ddc* transcription following infection (Figure 3.8A,B). Furthermore, heat shock induction of Fos and Jun expression did not activate *Ddc* transcription and expression of a dominant negative form of Fos or Jun, did not eliminate *Ddc* immune induction (Figure 3.8C,D). Most importantly, *Tak1* mutant flies induce *Ddc* transcription normally (Figure 3.9A). Taken together, these results suggest that the JNK pathway is not responsible for *Ddc* transcriptional induction. Although AP-1 can bind its putative binding site upstream of *Ddc in vitro*, our genetic data suggest that AP-1 does not induce *Ddc* transcription *in vivo*. Therefore, we conclude that a transcription factor other than AP-1 binds to this site to activate *Ddc* transcription.

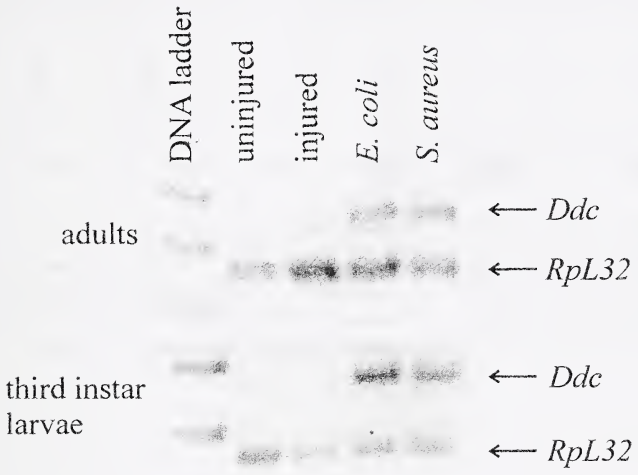
We have provided extensive evidence that the JNK pathway and its transcription factor AP-1 are not involved in the immune induction of *Ddc* transcription. The ERK pathway has been implicated in the induction of *Ddc* in the embryonic wound response, and treatment with a universal MAPK inhibitor reduces *Ddc* induction (28), suggesting that a MAPK is important for *Ddc* transcription. *Ddc* was immune inducible in the hypomorphic mutant of Drosophila ERK, *rl*^{1} (Figure 3.9B), suggesting that the ERK pathway is not responsible for *Ddc* immune induction. However, analysis of a null *rl* mutant would be necessary to confirm this observation.

The p38 MAPK pathway has been implicated in attenuation of the Drosophila immune response (29). A null allele of *p38a*, *Mpk2*^{1}, did not affect immune induction of *Ddc* transcription. No mutant for *p38b* has been identified, but *Ddc* transcription is induced normally in two lines bearing P-element insertions immediately upstream of the *p38b* gene. However, these insertions likely do not eliminate p38b function, and the creation of a null allele of *p38b* would be useful. Interestingly, the only mutant we identified that affected *Ddc* transcriptional induction was *p38c*^{KG05834}, which carries a P-element insertion in the coding sequence of an uncharacterised member of the p38 MAPK family, p38c (Figure 3.10A). The *p38c* gene is located immediately downstream of *p38a*, and likely arose by gene duplication (60). Four ESTs for *p38c* were detected in infected larval fat body cells (62). We have confirmed that this gene is expressed and that transcription of p38c is lost in *p38c*^{KG05834} (Figure 3.10B). Furthermore, the lack of *p38c* expression correlates with the loss of *Ddc* immune induction (Figure 3.10A). This suggests that p38c MAPK has a functional role in the *Drosophila* innate immune response.

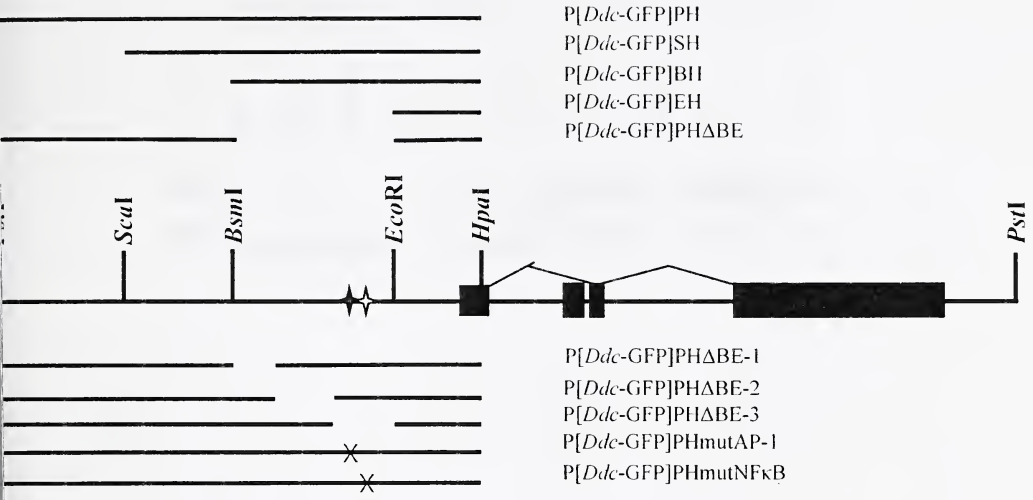
The identification of yet another signalling pathway that participates in the immune induction of gene transcription, points to the evolutionary significance of innate immunity. The p38c MAPK pathway elicits its response through the activation of an unknown transcription factor, which acts through the conserved AP-1 site upstream of the *Ddc* transcription start site. Transcription of p38c is not immune inducible (Figure 3.10D), suggesting that p38c MAPK is post-transcriptionally activated. It has been suggested that p38c cannot be activated by phosphorylation by a MAPKK due to the mutation that converts the TGY dual phosphorylation motif to TDH (61). However, a

clear role for p38c in *Ddc* immune induction has been demonstrated although the mechanism of p38c activation is still to be elucidated. It has also been suggested that the amino acid substitutions within the catalytic domain may render p38c non-functional as a kinase (61). However, further work is required to ascertain the catalytic activity of p38c and to delineate the other components of the p38c signalling pathway. Further analysis of MAPKKKs, MAPKKs and alternate transcription factors is essential to developing an understanding of how p38c induces *Ddc* transcription.

The importance of DDC to neuronal production of DA is well established. During development, DDC produces the DA that is required for melanin and sclerotin production (41,42). Tanning of the pupal case at pupariation and the hardening and darkening of the adult cuticle following eclosion are the most obvious manifestations of dopamine (DA) metabolism. *Ddc* transcription is also induced in the Drosophila embryonic wound response, at the wound site, to produce a melanin plug (28). Melanotic encapsulation of the parasitic wasp egg also requires DDC (67). In addition to these well established roles for DDC, here, we confirm a new role for *Ddc* in the Drosophila innate immune response to bacterial infection. *Ddc* transcription in the epidermis will increase the production of DA. The DA is most likely metabolized to produce reactive quinones that are toxic to invading bacteria. Presumably, DA is metabolized by the enzymes responsible for the production of sclerotin since no melanin deposits are evident within the epidermis. Consistent with this prediction, the gene, *black*, whose protein product is an enzyme involved in NBAD synthesis (68-70), is also upregulated in the Drosophila immune response (48). Future research will reveal the extent of the antimicrobial role of quinone production in the innate immune response.



igure 3.1: Immune induction of *Ddc* transcription following septic injury. Semi-uantitative RT-PCR was carried out on RNA obtained from adult flies and mid-third nstar larvae. Gene- specific transcripts in this and subsequent figures were separated on 2% agarose gel and are shown with a co-reversed transcribed and PCR amplified *RpL32* oading control. Transcripts from organisms septically injured with *E. coli* (lane 4) or *S. ureus* (lane 5) are compared to aseptically injured (lane 3) or uninjured organisms (lane). In this and subsequent figures, 1 kb Plus DNA ladder (Invitrogen) was used as a size tandard.



gure 3.2: Schematic diagram of the P[*Ddc*-GFP] reporter constructs used in this study. e epidermal-specific splice pattern shown above the line is situated on the 7.6 kb *Pst*I striction fragment that is sufficient for proper developmental expression of *Ddc*. The cond exon is neural-specific. The sequence that drives GFP expression is depicted in lines above and below the diagram of the *Ddc* genomic region. Relevant restrictions zyme sites, used to create the larger deletions, are shown. The black and white stars resent the conserved AP-1 and NFκB sites respectively, X's indicate an induced tation. The P[*Ddc*-GFP]PHmutNFκB contains a 5 bp mutation in the putative NFκB ding site and the P[*Ddc*-GFP]PHmutAP-1 construct contains a 5 bp mutation in the tative AP-1 site.

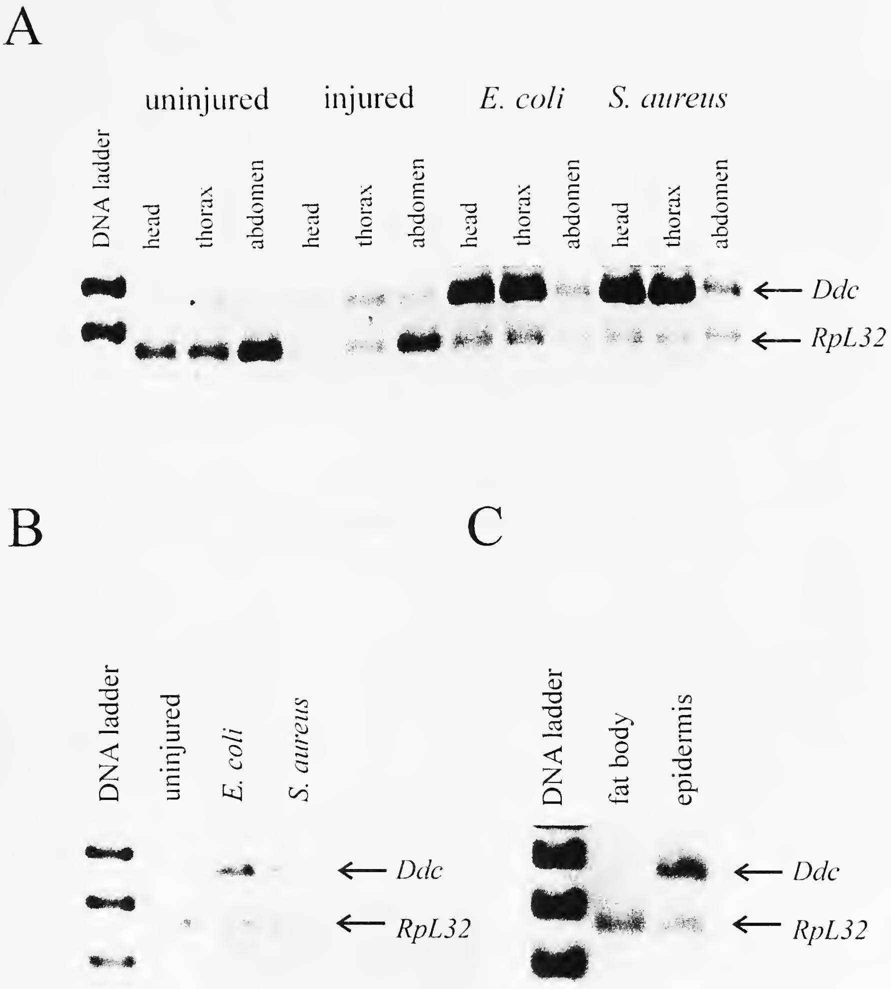


Figure 3.3: *Ddc* transcription is induced in the epidermis. A. Semi-quantitative RT-PCR from RNA isolated from heads, thorax and abdomens of uninjured, aseptically injured, and septically injured adult flies. B. RT-PCR on RNA isolated from mid-third instar dom^{K08108} larvae infected with *E. coli* or *S. aureus*. C. RT-PCR on RNA isolated from fat body and epidermal tissues dissected from mid-third instar larvae infected with *E. coli* or *S. aureus*.

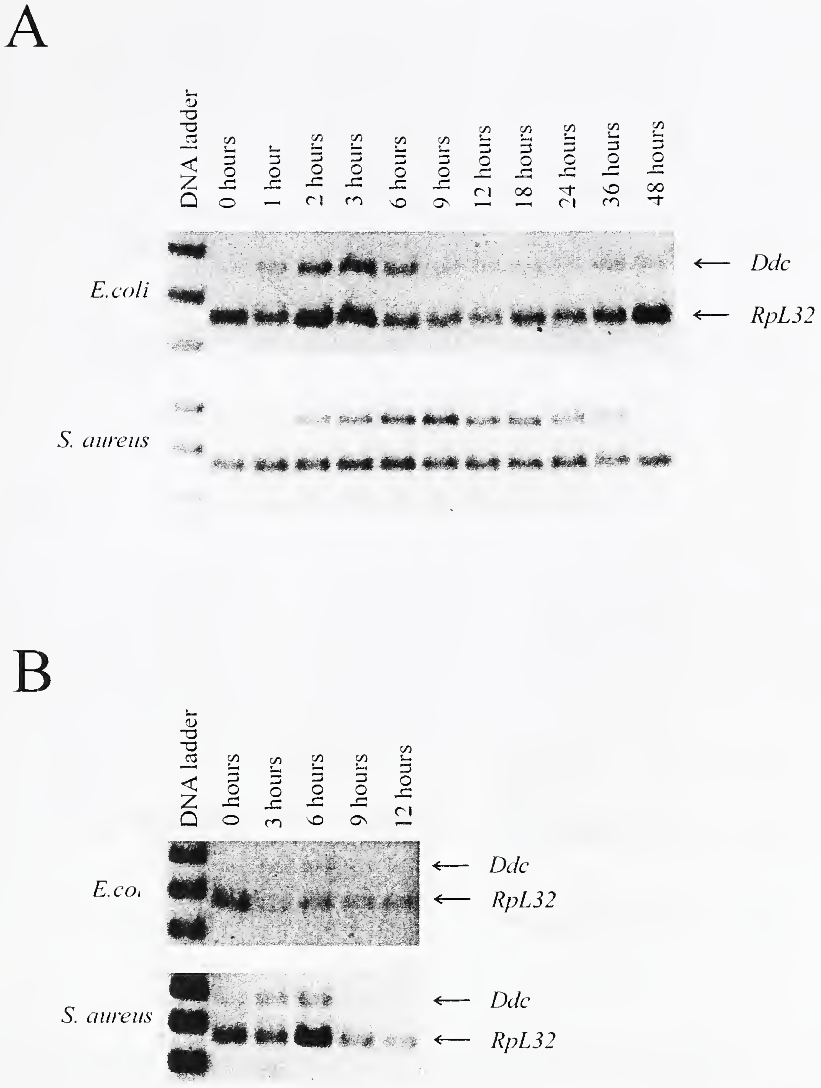


Figure 3.4: Induction of *Ddc* transcription in adults septically injured with live (A) or heat-killed (B) *E. coli* or *S. aureus*.

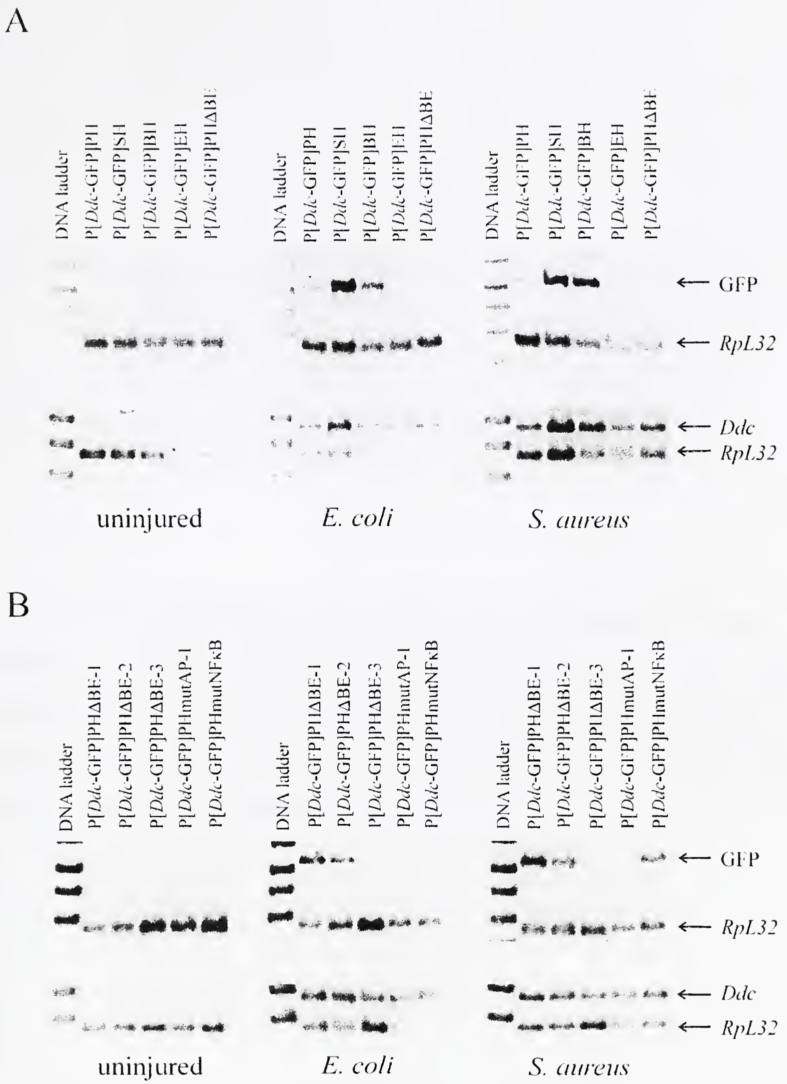


Figure 3.5: Mapping the region upstream of *Ddc* that is required for immune inducibility. Semi-quantitative RT-PCR of *Ddc*-GFP and endogenous *Ddc* transcripts was carried out on RNA isolated from adult flies bearing GFP reporter genes. *Ddc*-GFP or *Ddc* transcripts were co-reverse transcribed and PCR amplified with an *RpL32* loading control. A. Deletions localizing the response element to the *Bsm*I to *Eco*RI region (Figure 3.2). B. The *Bsm*I to *Eco*RI region contains a conserved AP-1 site that is necessary for immune inducibility of *Ddc*.

```
). melanogaster   tg-tttcgagtgactcataat----tggGGGATTCCtgacg
). Simulans       tg-tttcgagtgactcatgtt-----ggGGGATTCCtgtcg
). sechellia      tg-tttcgagtgactcatgat-----ggGGGATTCCtgtcg
). yakuba         tg-tttcgagtgactcatgat-----ggGGGATTCCcgacg
). erecta         tg-ttacgagtgactcatgat-----ggGGGATTCCtgacg
). ananassae      tg-gattttgtgactcatgat-----ggGGGATTCCtg---
). pseudoobscura  gg-tgtgacgtgactcatgac-----ggGGGATTCCtg---
). persimilis     gg-tgtgacgtgactcatgac-----ggGGGATTCCtg---
). willistoni     tgaccacttgtgattcatcacc-tg-tgGGGATTCCt----
). grimshawi      tg-caacatgtgactcataagcgctgggGGAATTCTt----
```

igure 3.6: Conserved AP-1 and NFκB sites lie within the region deleted in P[*Ddc*-FP]BE-3. Alignment of a portion of the *Ddc* region deleted in P[*Ddc*-GFP]BE-3 from 0 different Drosophila species reveals a conserved AP-1 (underlined and bold) and a FκB site (capital letters and bold) embedded in a less conserved area. Deviations from e *D. melanogaster* sequence are italicized.

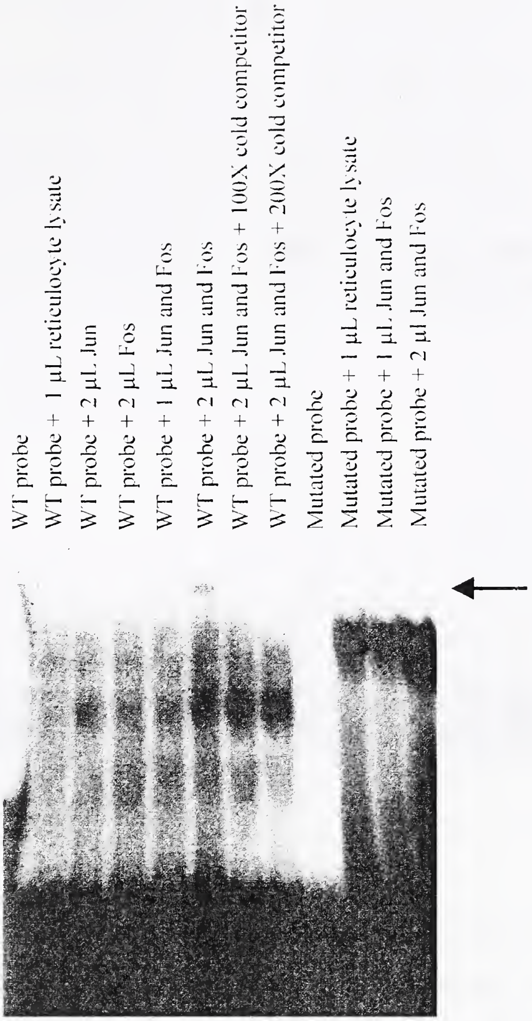
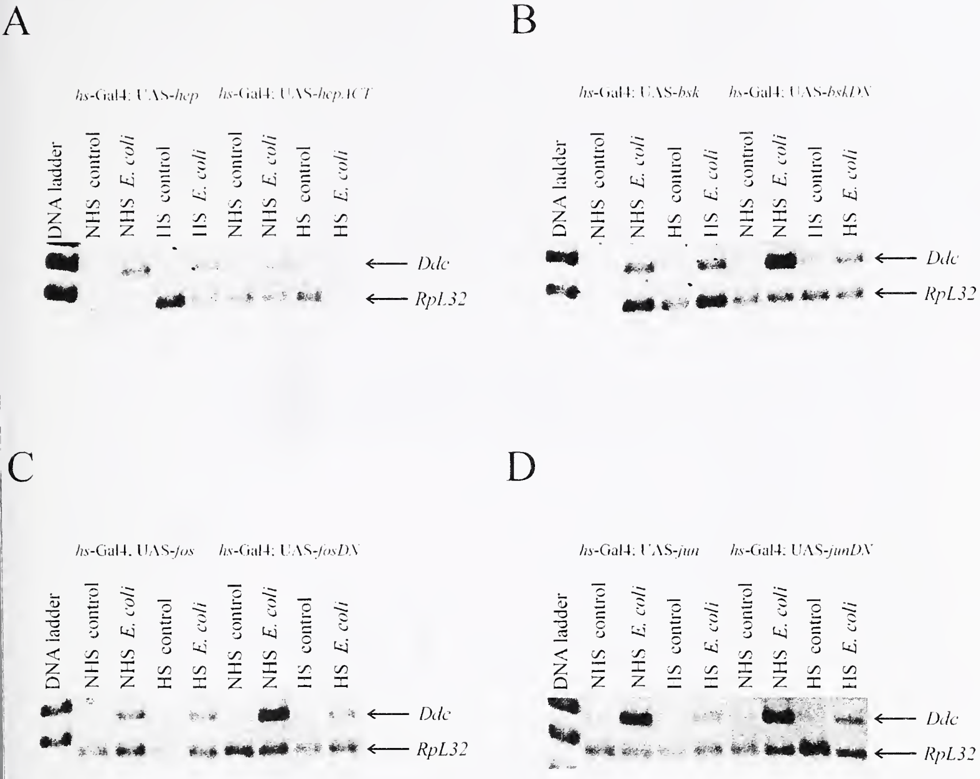
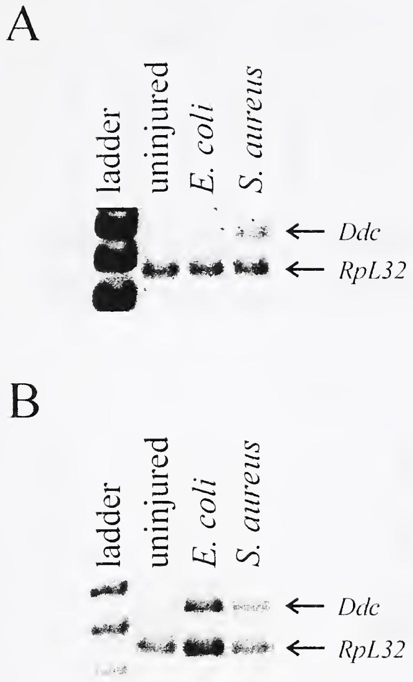

WT probe
WT probe + 1 µL reticulocyte lysate
WT probe + 2 µL Jun
WT probe + 2 µL Fos
WT probe + 1 µL Jun and Fos
WT probe + 2 µL Jun and Fos
WT probe + 2 µL Jun and Fos + 100X cold competitor
WT probe + 2 µL Jun and Fos + 200X cold competitor
Mutated probe
Mutated probe + 1 µL reticulocyte lysate
Mutated probe + 1 µL Jun and Fos
Mutated probe + 2 µl Jun and Fos

Figure 3.7: The Jun/Fos complex binds to the *Ddc* AP-1 site *in vitro*. An electrophoretic mobility shift assay using recombinant Drosophila Jun and Fos was carried out. Lanes 1-8 and 9-12 contain wild-type and mutant probe respectively. (See the Materials and Methods for probe preparation). Lanes 2 and 10 contain probes incubated with the reticulocyte lysate. Jun (lane 3) or Fos (lane 4) were added alone or in varying amounts together (lanes 5, 6, 11 and 12). Cold competitor was added to the reactions shown in lanes 7 and 8.



igure 3.8: Immune induction of *Ddc* is unaffected in uninjured (control) and *E. coli* fected flies following heat shock (HS) (one hour heat shock followed by one hour covery) or left untreated (NHS). A. A wild-type HEP (*hs*-Gal4; UAS-*hep*) or a nstitutively active form of HEP (*hs*-Gal4; UAS-*hepACT*) was expressed; B. A wild-pe BSK (*hs*-Gal4; UAS-*bsk*) or a dominant negative form of BSK (*hs*-Gal4; UAS-*kDN*) was expressed; C wild-type Fos (*hs*-Gal4; UAS-*fos*) or a dominant negative form Fos (*hs*-Gal4; UAS-*fosDN*) was expressed; D. wild-type Jun (*hs*-Gal4; UAS-*jun*) or a minant negative form of Jun (*hs*-Gal4-*junDN*) was expressed. *S. aureus* infection imics the results following *E. coli* infection (data not shown)



igure 3.9: Immune induction of *Ddc* is unaffected in a JNK loss of function and an ERK ypomorphic mutant. *Ddc* RT-PCR on RNA obtained from uninjured and septically jured A. *Tak1* or B. *rl^1* mutant flies.

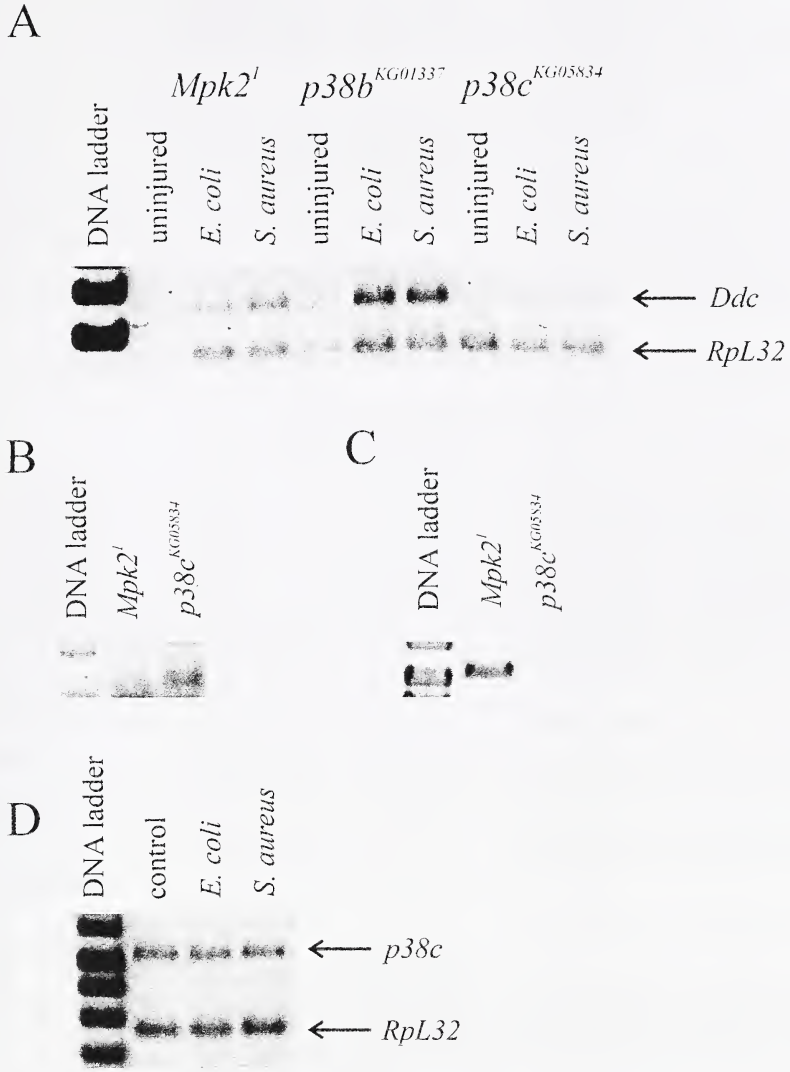


Figure 3.10: p38c MAPK is required for immune induction of *Ddc*. A. *Ddc* RT-PCR on RNA obtained from uninjured and infected $Mpk2^{1}$, $p38b^{KG01337}$, and $p38c^{KG05834}$ adult flies. B. *p38a* transcript analysis in $Mpk2^{1}$ and $p38c^{KG05834}$ mutant flies. C. *p38c* transcript analysis in $Mpk2^{1}$ and $p38c^{KG05834}$ mutant flies. D. *p38c* transcription is not immune inducible.

Genotype	**Source**	**Use/nature of allele**
yw	Bloomington stock #1495	wild-type control
dom^{K08108}	Bloomington stock #10767	P-element insertion in *dom*, which causes loss of haemocytes
Ddc^{ts2} *cn/CyO*	Bloomington stock #3004	temperature sensitive *Ddc* mutant
Df(2L)130 pr cn/CyO	Bloomington stock #2349	Deletion removes the *Ddc* gene
rdo hk Ddc^{n7} *pr cn/CyO*	(52)	Null allele of *Ddc*
Act5C-Gal4	Bloomington stock #3954	Actin promoter expressing Gal4
yw; P[*Ddc*-RNAi]pWIZ	This study	expression of a *Ddc* RNAi construct
yw; P[*Ddc*-RNAi]pSymp	This study	expression of a *Ddc* RNAi construct
w;hs-Gal4	Bloomington stock #1799	heat shock promoter expressing Gal4
yw; P[*Ddc*-GFP]PH	(71)	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]SH	(71)	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]BH	(71)	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]EH	(71)	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHΔBE	This study	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHΔBE1	This study	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHΔBE2	This study	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHΔBE3	This study	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHmutNFκB	This study	*Ddc* reporter construct line
yw; P[*Ddc*-GFP]PHmutAP-1	This study	*Ddc* reporter construct line
Act5C-Gal4	Bloomington stock #3954	Actin promoter expressing Gal4
w;hs-Gal4	Bloomington stock #1799	heat shock expression of Gal4
UAS-*hep*	Bloomington stock #6406, #9307, and #9308	expression of a wild-type HEP
UAS-*bsk*	Bloomington stock #6407 and #9310	expression of a wild-type BSK
UAS-*jun*	Bloomington stock #7216	expression of a wild-type Jun
UAS-*fos*	Bloomington stock #7213	expression of a wild-type Fos
UAS-*hepACT*	Bloomington stock #9305 and #9306	expression of a constitutively active HEP
UAS-*bskDN*	Bloomington stock #6409 and #9311	expression of a dominant negative BSK
UAS-*junDN*	Bloomington stock #7217	expression of a dominant

	and #7218	negative Jun
UAS-*fosDN*	Bloomington stock #7214 and #7215	expression of a dominant negative Fos
yw; Tak1	(14)	IMD and JNK pathway deficient
yw; ird5^{KG08072}	Bloomington stock #14684	P-element insertion in the first exon of *ird5*
yw; ird5^{EY02434}	Bloomington stock #19825	P-element insertion in the first exon of *ird5*
yw; Rel^{EY08061}	Bloomington stock #20016	P-element insertion in the first exon of *Rel*
mwh^{1} *kni*$^{ri-1}$ *snk*^{4} *red*^{1} *e Tl*^{3} *ca/Ms(3) R24/TM3, Sb*	Bloomington stock #3338	Balanced gain of function *Tl* mutant
rl^{1}	Bloomington stock #386	spontaneous hypomorphic mutation in ERK
Mpk2^{1}	Bloomington stock #8822	deletion of *p38a* MAPK gene
p38b^{KG02737}	Bloomington stock #12905	P-element insertion in 5' region of the *p38b* MAPK gene
p38b^{KG01337}	Bloomington stock #14364	P-element insertion in 5' region of the *p38b* MAPK gene
yw; p38c^{KG05834}	Bloomington stock #14126	P-element insertion in the coding sequence of the *p38c* MAPK gene

Table 3.1: Fly stocks used in this study. Where applicable, the source of the stock and the nature of the mutation or the purpose of the stock are given. *CyO = In (2LR)O, Cy dp*^{lvI} *pr cn*^{2}.

				Percent lethality after:		
	Genotype	**infection**	**hs?**	**24 hours**	**48 hours**	**72 hours**
A	*Ddc*^{ts2} *cn/ Ddc*^{n7}	*E. coli*	no	2 (1/50)	6 (3/50)	10 (5/50)
		S. aureus	no	10 (6/60)	71.7 (43/60)	73.3 (44/60)
	Ddc^{ts2} *or Ddc*^{n7}*/ CyO*	*E. coli*	no	3.3 (1/30)	6.7 (2/30)	6.7 (2/30)
		S. aureus	no	26.7 (8/30)	80 (24/30)	83.3 (25/30)
B	*Ddc*^{ts2}*cn/ Df(2L)130*	*E. coli*	no	5 (1/20)	10 (2/20)	15 (3/20)
		S. aureus	no	6.4 (2/31)	58.1 (18/31)	80.6 (25/31)
	Ddc^{ts2} *or Df(2L)130/CyO*	*E. coli*	no	0 (0/30)	3.3 (1/30)	13.3 (4/30)
		S. aureus	no	13.3 (4/30)	83.3 (25/30)	90 (27/30)
C	*hs-Gal4; Ddc*-RNAi *(pWIZ)*	*E. coli*	no	9.7 (12/123)	18.7 (23/123)	15.4 (19/123)
		E. coli	yes	3.3 (2/60)	6.7 (4/60)	13.3 (8/60)
		S. aureus	no	40.2 (41/102)	86.3 (88/102)	91.1 (93/102)
		S. aureus	yes	30 (18/60)	66.7 (40/60)	86.7 (52/60)

Table 3.2: Reduction of DDC activity does not affect survival following septic injury. Survival of *Ddc* mutant flies and *Ddc*-RNAi expressing flies after infection with *E. coli* or *S. aureus* was tracked for three days. A, B. Survival of the straight-winged heteroallelic *Ddc* mutants was compared to the curly-winged wild-type siblings following septic injury with *E. coli* or *S. aureus*. C. Survival of *hs*-Gal4; P[*Ddc*-RNAi]pWIZ flies following heat shock (hs) (1 hour at 37°C, 1 hour recovery) and no heat shock (control) after septic injury with *E. coli* or *S. aureus*.

References

1. Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. and Ezekowitz, R.A. (1999). Phylogenetic perspectives in innate immunity. Science (New York, NY. 284: 1313-1318.
2. Lemaitre, B. and Hoffmann, J. (2007). The host defense of *Drosophila melanogaster*. Annu Rev Immunol. 25: 697-743.
3. Imler, J.L. and Bulet, P. (2005). Antimicrobial peptides in Drosophila: Structures, activities and gene regulation. Chem Immunol Allergy. 86: 1-21.
4. Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., et al. (1995). A recessive mutation, *immune deficiency* (*imd*), defines two distinct control pathways in the Drosophila host defense. Proc Natl Acad Sci U S A. 92: 9465-9469.
5. Corbo, J.C. and Levine, M. (1996). Characterization of an immunodeficiency mutant in Drosophila. Mech Dev. 55: 211-220.
6. Levashina, E.A., Ohresser, S., Lemaitre, B. and Imler, J.L. (1998). Two distinct pathways can control expression of the gene encoding the Drosophila antimicrobial peptide metchnikowin. J Mol Biol. 278: 515-527.
7. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., et al. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 86: 973-983.
8. Rutschmann, S., Kilinc, A. and Ferrandon, D. (2002). Cutting edge: The toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. J Immunol. 168: 1542-1546.
9. Meng, X., Khanuja, B.S. and Ip, Y.T. (1999). Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. Genes & development. 13: 792-797.
10. Manfruelli, P., Reichhart, J.M., Steward, R., Hoffmann, J.A., et al. (1999). A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. The EMBO journal. 18: 3380-3391.
11. Rutschmann, S., Jung, A.C., Hetru, C., Reichhart, J.M., et al. (2000). The Rel protein DIF mediates the antifungal but not the antibacterial host defense in Drosophila. Immunity. 12: 569-580.

12. Hedengren, M., Asling, B., Dushay, M.S., Ando, I., et al. (1999). Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol Cell. 4: 827-837.

13. Boutros, M., Agaisse, H. and Perrimon, N. (2002). Sequential activation of signaling pathways during innate immune responses in Drosophila. Developmental cell. 3: 711-722.

14. Vidal, S., Khush, R.S., Leulier, F., Tzou, P., et al. (2001). Mutations in the Drosophila *dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes & development. 15: 1900-1912.

15. Silverman, N., Zhou, R., Erlich, R.L., Hunter, M., et al. (2003). Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J Biol Chem. 278: 48928-48934.

16. Glise, B., Bourbon, H. and Noselli, S. (1995). *hemipterous* encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. Cell. 83: 451-461.

17. Riesgo-Escovar, J.R., Jenni, M., Fritz, A. and Hafen, E. (1996). The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. Genes & development. 10: 2759-2768.

18. Kockel, L., Homsy, J.G. and Bohmann, D. (2001). Drosophila AP-1: Lessons from an invertebrate. Oncogene. 20: 2347-2364.

19. Zhang, K., Chaillet, J.R., Perkins, L.A., Halazonetis, T.D., et al. (1990). Drosophila homolog of the mammalian jun oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc Natl Acad Sci U S A. 87: 6281-6285.

20. Perkins, K.K., Admon, A., Patel, N. and Tjian, R. (1990). The Drosophila Fos-related AP-1 protein is a developmentally regulated transcription factor. Genes & development. 4: 822-834.

21. Ramet, M., Lanot, R., Zachary, D. and Manfruelli, P. (2002). JNK signaling pathway is required for efficient wound healing in Drosophila. Dev Biol. 241: 145-156.

22. Galko, M.J. and Krasnow, M.A. (2004). Cellular and genetic analysis of wound healing in Drosophila larvae. PLoS biology. 2: E239.

23. Bosch, M., Serras, F., Martin-Blanco, E. and Baguna, J. (2005). JNK signaling pathway required for wound healing in regenerating Drosophila wing imaginal discs. Dev Biol. 280: 73-86.

24. Zettervall, C.J., Anderl, I., Williams, M.J., Palmer, R., et al. (2004). A directed screen for genes involved in Drosophila blood cell activation. Proc Natl Acad Sci U S A. 101: 14192-14197.

25. Kallio, J., Leinonen, A., Ulvila, J., Valanne, S., et al. (2005). Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. Microbes and infection / Institut Pasteur. 7: 811-819.

26. Delaney, J.R., Stoven, S., Uvell, H., Anderson, K.V., et al. (2006). Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways. The EMBO journal. 25: 3068-3077.

27. Kim, L.K., Choi, U.Y., Cho, H.S., Lee, J.S., et al. (2007). Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. PLoS biology. 5: e238.

28. Mace, K.A., Pearson, J.C. and McGinnis, W. (2005). An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science (New York, NY. 308: 381-385.

29. Han, Z.S., Enslen, H., Hu, X., Meng, X., et al. (1998). A conserved p38 mitogen-activated protein kinase pathway regulates Drosophila immunity gene expression. Mol Cell Biol. 18: 3527-3539.

30. Martin-Blanco, E. (2000). p38 MAPK signalling cascades: Ancient roles and new functions. Bioessays. 22: 637-645.

31. Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., et al. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity. 13: 737-748.

32. Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., et al. (1998). A *drosomycin*-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. The EMBO journal. 17: 1217-1227.
33. Bidla, G., Lindgren, M., Theopold, U. and Dushay, M.S. (2005). Hemolymph coagulation and phenoloxidase in Drosophila larvae. Dev Comp Immunol. 29: 669-679.
34. Rizki, T.M., Rizki, R.M. and Bellotti, R.A. (1985). Genetics of a Drosophila phenoloxidase. Mol Gen Genet. 201: 7-13.
35. Meister, M. (2004). Blood cells of Drosophila: Cell lineages and role in host defence. Curr Opin Immunol. 16: 10-15.
36. Meister, M. and Lagueux, M. (2003). Drosophila blood cells. Cellular microbiology. 5: 573-580.
37. Scherfer, C., Karlsson, C., Loseva, O., Bidla, G., et al. (2004). Isolation and characterization of hemolymph clotting factors in *Drosophila melanogaster* by a pullout method. Curr Biol. 14: 625-629.
38. Karlsson, C., Korayem, A.M., Scherfer, C., Loseva, O., et al. (2004). Proteomic analysis of the Drosophila larval hemolymph clot. J Biol Chem. 279: 52033-52041.
39. Nappi, A.J. and Ottaviani, E. (2000). Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays. 22: 469-480.
40. Bidla, G., Dushay, M.S. and Theopold, U. (2007). Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J Cell Sci. 120: 1209-1215.
41. Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet. 24: 127-222.
42. Andersen, S.O., Hojrup, P. and Roepstorff, P. (1995). Insect cuticular proteins. Insect Biochem Mol Biol. 25: 153-176.
43. Walter, M.F., Zeineh, L.L., Black, B.C., McIvor, W.E., et al. (1996). Catecholamine metabolism and *in vitro* induction of premature cuticle melanization in wild type and pigmentation mutants of *Drosophila melanogaster*. Arch Insect Biochem Physiol. 31: 219-233.

44. Nappi, A.J., Carton, Y. and Vass, E. (1992). Reduced cellular immune competence of a temperature-sensitive *Dopa decarboxylase* mutant strain of *Drosophila melanogaster* against the parasite *Leptopilina boulardi*. Comp Biochem Physiol. 101: 453-460.
45. Kim, M.H., Joo, C.H., Cho, M.Y., Kwon, T.H., et al. (2000). Bacterial-injection-induced syntheses of N-beta-alanyldopamine and Dopa decarboxylase in the hemolymph of coleopteran insect, *Tenebrio molitor* larvae. Eur J Biochem. 267: 2599-2608.
46. Schachter, J., Perez, M.M. and Quesada-Allue, L.A. (2007). The role of N-beta-alanyldopamine synthase in the innate immune response of two insects. J Insect Physiol: 1188-1197.
47. Boutros, M., Agaisse, H. and Perrimon, N. (2002). Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev Cell. 3: 711-722.
48. Irving, P., Troxler, L., Heuer, T.S., Belvin, M., et al. (2001). A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci U S A. 98: 15119-15124.
49. De Gregorio, E., Spellman, P.T., Rubin, G.M. and Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci U S A. 98: 12590-12595.
50. Braun, A., Hoffmann, J.A. and Meister, M. (1998). Analysis of the Drosophila host defense in *domino* mutant larvae, which are devoid of hemocytes. Proc Natl Acad Sci U S A. 95: 14337-14342.
51. Lazareva, A.A., Roman, G., Mattox, W., Hardin, P.E., et al. (2007). A role for the adult fat body in Drosophila male courtship behavior. PLoS genetics. 3: e16.
52. Wright, T.R., Steward, R., Bentley, K.W. and Adler, P.N. (1981). The Genetics of Dopa decarboxylase in *Drosophila melanogaster* III. Effect of a temperature sensitive Dopa decarboxylase Deficient mutation of female fertility. Developmental Genetics. 2: 223-235.
53. Wright, T.R., Bewley, G.C. and Sherald, A.F. (1976). The genetics of Dopa decarboxylase in *Drosophila melanogaster*. II. Isolation and characterization of

dopa-decarboxylase-deficient mutants and their relationship to the alpha-methyl-dopa-hypersensitive mutants. Genetics. 84: 287-310.

54. Kent, W.J. (2002). BLAT--the BLAST-like alignment tool. Genome research. 12: 656-664.

55. Schug, J. (2003). Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence. In: Baxevanis, A.D. (ed.), Current Protocols in Bioinformatics. J. Wiley and Sons.

56. Rutschmann, S., Jung, A.C., Zhou, R., Silverman, N., et al. (2000). Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nat Immunol. 1: 342-347.

57. Silverman, N., Zhou, R., Stoven, S., Pandey, N., et al. (2000). A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes & development. 14: 2461-2471.

58. Lu, Y., Wu, L.P. and Anderson, K.V. (2001). The antibacterial arm of the Drosophila innate immune response requires an IkappaB kinase. Genes & development. 15: 104-110.

59. Biggs, W.H., 3rd, Zavitz, K.H., Dickson, B., van der Straten, A., et al. (1994). The Drosophila *rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway. Embo J. 13: 1628-1635.

60. Manning, G. (2001). Novel kinase: p38c. Personal communication to FlyBase. http://flybase.bio.indiana.edu/reports/FBrf0139874.html

61. Craig, C.R. (2003). *p38c* gene. Personal communication to FlyBase. http://flybase.bio.indiana.edu/reports/FBrf0182691.html

62. Carlson, J. (2005). FlyTag EST project. Personal communication to FlyBase. http://flybase.bio.indiana.edu/reports/FBrf0188689.html

63. Craig, C.R., Fink, J.L., Yagi, Y., Ip, Y.T., et al. (2004). A Drosophila p38 orthologue is required for environmental stress responses. EMBO reports. 5: 1058-1063.

64. Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the Drosophila embryo. Dev Biol. 246: 57-67.

65. Perkins, K.K., Dailey, G.M. and Tjian, R. (1988). Novel Jun- and Fos-related proteins in Drosophila are functionally homologous to enhancer factor AP-1. The EMBO journal. 7: 4265-4273.
66. Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell. 103: 239-252.
67. Nappi, A.J., Carton, Y. and Vass, E. (1992). Reduced cellular immune competence of a temperature-sensitive *Dopa decarboxylase* mutant strain of *Drosophila melanogaster* against the parasite *Leptopilina boulardi*. Comp Biochem Physiol B. 101: 453-460.
68. Weber, J.P., Bolin, R.J., Hixon, M.S. and Sherald, A.F. (1992). Beta-alanine transaminase activity in *black* and *suppressor of black* mutations of *Drosophila melanogaster*. Biochim Biophys Acta. 1115: 181-186.
69. Hodgetts, R.B. (1972). Biochemical characterization of mutants affecting the metabolism of β-alanine in Drosophila. Journal of insect physiology. 18: 937-947.
70. Phillips, A.M., Smart, R., Strauss, R., Brembs, B., et al. (2005). The Drosophila *black* enigma: The molecular and behavioural characterization of the *black1* mutant allele. Gene. 351: 131-142.
71. Davis, M.M., Yang, P., Chen, L., O'Keefe, S.L., et al. (2007). The orphan nuclear receptor DHR38 influences transcription of the *Dopa decarboxylase* gene in epidermal and neural tissues of *Drosophila melanogaster*. Genome. 50: 1049-1060.

Chapter 4: General conclusions

I have analyzed the regulation of tyrosine hydroxylase (TH) and Dopa decarboxylase (DDC) in an attempt to understand how systemic signals can elicit cell-, protein-, and gene-specific effects. I have divided this conclusion into two parts; the first deals with hormonal regulation of cuticular tanning following eclosion, the second deals with immune induction of *Ddc* transcription.

Part 1: Hormonal regulation of cuticular tanning following eclosion

To understand how hormones regulate development, I examined how the ecdysis neuropeptide cascade controlled the precise timing of post-eclosion developmental events. This project was inspired by the observation that eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) genetic ablation cell knock-outs (EH-KO and CCAP-KO respectively) have the same adult phenotype as *bursicon* (*burs*) and *rickets* (*rk*) mutants (1-4). Such flies fail to expand their cuticle and wings post-eclosion, and exhibit delays in cuticular tanning. Tanning is a convenient visible cue of post-eclosion development that requires metabolites of dopamine (DA) (5), which is produced by the actions of TH, encoded by *pale* (*ple*) (6), and DDC, encoded by *Ddc* (7,8). The delay in tanning in EH-KO, CCAP-KO, *burs* and *rk* flies suggested that the ecdysis neuropeptide cascade somehow regulated the production of DA.

Three observations provided support for this hypothesis:

1. Flies neck-ligated at eclosion fail to tan whereas those ligated 30 minutes after eclosion tan normally (9). This suggests that a neuropeptide is released immediately after eclosion that triggers the precise onset of tanning. Consistent with this prediction, Bursicon has long been known as the post-eclosion hormone required for tanning (10). This suggests that Bursicon may regulate one of the enzymes required for DA production.

2. Injection of 8-Br-cAMP rescues the tanning defect in *rk* mutants and flies neck-ligated at eclosion (4,11). Both CCAP and Bursicon/RK signalling activate adenylyl cyclase, which causes a corresponding increase in cAMP (12,13). This suggests that either CCAP or Bursicon may regulate tanning through an increase in cAMP.

3. The major contributor to Drosophila TH activation is protein kinase A (PKA), which phosphorylates Ser32 (14). PKA is activated by an increase in cAMP. Since both CCAP and Bursicon signalling cause an increase in cAMP levels, either hormone may cause activation of TH to induce tanning post eclosion.

To determine how the ecdysis neuropeptide cascade controlled tanning, I undertook experiments that examined the transcription of *ple* and *Ddc*, and the protein, phosphorylation and activity levels of TH.

The results of these experiments led to several conclusions:

1. Transcription of *ple* and *Ddc* does not regulate the precise onset of post-eclosion tanning. Transcripts for *ple* accumulate prior to eclosion, and remain high until long after post-eclosion tanning has occurred. *Ddc* transcripts accumulate early and their levels begin to drop before eclosion.

2. CCAP regulates the translation of TH through an increase in cAMP. Levels of TH protein and activity rise prior to eclosion, when TH activity is necessary for pigmentation of the pharate adult bristles and epidermis. Levels drop prior to eclosion and rise thereafter. Since *ple* transcripts are present during the entire period of TH protein and activity fluctuation at eclosion, translational control must be exerted on the *ple* transcripts. TH protein begins to accumulate within 1 hour, and is transiently activated by phosphorylation from 1.5 to 3 hours after eclosion, corresponding to the time when tanning occurs. Neck-ligation of flies at eclosion blocks phosphorylation, but not accumulation of TH. Therefore, a factor released before eclosion likely regulates translation of TH. A role for CCAP in the control of TH translation was inferred from the observation that CCAP-KO flies completely fail to accumulate TH after eclosion and that translation of TH is restored in CCAP-KO flies by injection of either CCAP or 8-Br-cAMP.

3. Bursicon/RK signalling controls the precise onset of tanning by causing the activation, by phosphorylation, of TH. Flies neck-ligated 30 minutes after eclosion phosphorylate TH normally, suggesting that a neuropeptide that is released after eclosion regulates phosphorylation. Since Ser32 phosphorylation is absent in *burs* mutants and reduced in *rk* mutants, Bursicon appears to be the neuropeptide responsible. This was

confirmed by the fact that injection of 8-Br-cAMP restored phosphorylation in *burs* and *rk* mutants as well as those that were neck-ligated at eclosion.

The data presented here demonstrate that tanning is regulated at a variety of levels. The first involves the transcription of *ple* and *Ddc*, since without accumulation of these transcripts, tanning would not occur. However, the precise onset of tanning following eclosion is regulated by the ecdysis neuropeptide cascade. First, CCAP release triggers the translation of TH, causing accumulation of TH in a timely fashion, immediately after eclosion. Then, after eclosion, Bursicon signalling, through RK, causes the phosphorylation and activation of TH that causes the peak of activity that is required for the rapid tanning of the adult cuticle.

Two questions arise from these data:

1. How is transcription of *ple* and *Ddc* regulated during metamorphosis?

Research has identified 17 Broad (BR) binding sites, representing all four isoforms (Z1-Z4) (15), within the first intron of *Ddc* (16). Using *Ddc*-LacZ reporter constructs, it was demonstrated that this intronic sequence is required for *Ddc* transcription in pharate adults (17). There are 4 isoforms of BR (15), of which one, BR-Z2, is responsible for *Ddc* transcriptional induction, along with ecdysone, at pupariation (17,18). Inconsistent results with fly lines bearing un-insulated *Ddc*-LacZ reporter constructs (19) prompted creation of a similar set of insulated *Ddc*-GFP constructs. These constructs contain ~2.6 kb of sequence upstream of the *Ddc* transcription start site. The 3' end of these constructs either terminates in the first exon of *Ddc* or at the end of the first intron. Semi-quantitative RT-PCR has confirmed that the first intron is required for pharate adult transcription of *Ddc* (data not shown), since deletion of this sequence completely eliminates *Ddc*-GFP expression. This suggests that the ecdysone response element (EcRE) upstream of the *Ddc* transcription start site is insufficient for transcriptional induction in pharate adults.

I have also created three *Ddc*-GFP constructs that each contain a deletion of a different third of the intron. Induction of reporter gene transcription occurs normally in pharate adults missing the first third of the intron. This suggests that this sequence is dispensable for *Ddc* transcription in pharate adults. Unfortunately, I was unable to obtain

transgenic flies bearing the other two constructs. These flies are essential to understanding how *Ddc* transcription is controlled in pharate adults, and efforts should be undertaken to obtain these transgenic organisms.

The mechanism of epidermal *ple* transcriptional regulation is completely unknown. To understand how it is transcriptionally regulated, I would create a series of *ple*-GFP reporter constructs to define the sequence elements within the promoter and the intron that are required. Alignment programs (20) and transcription factor databases (21) will aide in helping to identify the transcription factors that are involved. Since *Ddc* transcription is regulated by both ecdysone and BR (17,18), and *ple* is expressed in the same tissues as *Ddc*, it would not be surprising if *ple* was regulated in the same manner.

Analysis of the temporal expression patterns of the BR isoforms suggests that the Z1 isoform is the best candidate for the regulator of transcription in pharate adults (22,23). Since no BR mutants survive until eclosion (15), RNAi is the best technique to use to determine which isoform may act to activate *ple* or *Ddc* transcription in pharate adults (24). Furthermore, all ecdysone receptor (EcR) mutants die before pharate adults express *ple* or *Ddc* (25). A dominant negative form of EcR can be expressed in pharate adults to eliminate EcR signalling (26). Heat shock induced replacement of specific isoforms and tracking of *ple* or *Ddc* transcription would allow us to determine which isoform of EcR was responsible for *ple* transcriptional induction.

2. How is TH translation regulated?

In Drosophila, translational regulation most often occurs by microRNA (miRNA) acting through multiple binding sites in the 3' untranslated region (UTR) of transcripts (27). Three miRNAs are predicted to regulate TH translation in Drosophila (28). These miRNAs include let-7, which has 2 putative binding sites, and mir-iab-4-3p and mir-iab-4-5p, which have one potential site each within the 3' untranslated of the *ple* transcript. It is conceivable that one or more of these miRNAs causes repression of TH translation prior to eclosion, through a miRNA-mediated mechanism.

To determine if this is the case, I would use a *ple*-Gal4 (29) to drive the expression of a reporter gene containing the *ple* 3' UTR. Attaching the 3' UTR of *ple* to a UAS driven reporter gene, such as GFP, will allow determination of the role of each

miRNA in repression of TH translation. Using site-directed mutagenesis, I could mutate the putative miRNA binding sites and track reporter gene expression at eclosion in flies bearing these constructs. Furthermore, ectopic expression of the individual miRNAs will allow for confirmation of their repressive effects.

RNA binding proteins are also required for proper association of mRNA with the RISC complex. Once such RNA binding protein, fragile X mental retardation protein 1 (FMR1) has been proposed to regulate translation through an RNAi-dependent mechanism by interacting with the RISC and using an associated miRNA to identify translational targets (27). Interestingly, Drosophila *fmr1* mutants have increased DA synthesis in the brain compared to wild-type flies (30). This is caused by increased expression of two enzymes, phenylalanine hydroxylase, and GTP cyclohydrolase. Phenylalanine hydroxylase (See Appendix 1) converts phenylalanine to tyrosine and an overproduction of this enzyme provides more substrate to TH that causes a corresponding increase in the amount of DA produced. An increase in the amount of GTP cyclohydrolase, which is responsible for production of the essential TH cofactor, tetrahydrobiopterin, will cause an increase in the activity of TH. As FMR1 appears to be involved in the regulation of two enzymes required for DA synthesis, it is plausible that it also plays a role in the translational control of TH. Interestingly, the ability of the human homolog of FMR1 to bind mRNA and repress translation is reduced by phosphorylation by PKA, *in vitro* (31). Thus, CCAP may act through cAMP to activate PKA, which can then relieve translation repression.

To determine if FMR1 plays a role in TH translational repression, I would first need to analyse TH activity in the *fmr1* mutants. I would also have to determine if *fmr1* is expressed in the epidermis. If *fmr1* is expressed in the epidermis and TH activity is increased in *fmr1* mutants, I could then characterize the ability of FMR1 to bind the 3' UTR of *ple* mRNA. To do this, I would immunoprecipitate FMR1 from crude extracts of organisms immediately prior to eclosion, and detect *ple* transcripts by RT-PCR. I could also analyse the ability of FMR1 to bind *ple* transcripts in CCAP-KO flies and CCAP-KO flies injected with 8-Br-cAMP using this method. Presumably, FMR1 would continuously be bound to *ple* transcripts in CCAP-KO flies, and injection of 8-Br-cAMP

should relieve this constitutive repression. Furthermore, ectopic expression of *fmr1* at eclosion should delay TH accumulation following eclosion.

Part 2: Induction of *Ddc* transcription in the innate immune response

This project was inspired by microarray studies that had identified *Ddc* as an immune response gene (32-34). Although *Ddc* had been implicated in the embryonic wound response (35) and the cellular response to parasitic wasp infestation (36), a role for *Ddc* in the immune response to bacterial infection had not previously been shown.

To determine if *Ddc* was, in fact, induced in the immune response, I septically injured third instar larvae and adult flies and analysed *Ddc* expression by quantitative RT-PCR. I confirmed that *Ddc* transcription was induced following bacterial infection. Transcriptional induction is transient, appearing two hours after septic injury and remaining high for 9 hours after infection with gram negative bacteria, and for 36 hours after gram positive infection. Subsequently, I undertook several experiments to characterize the temporal and spatial expression of *Ddc* in the immune response.

From these experiments, several conclusions can be made:

1. A conserved AP-1 binding site upstream of the *Ddc* transcription start site is required for induction of *Ddc* following bacterial infection. AP-1 can bind this site *in vitro*, but ectopic expression of JNK pathway components had no effect on immune induction of *Ddc*. Furthermore, *Tak1* mutants, which fail to activate the immune Imd or JNK signalling pathways (37,38), induce *Ddc* transcription normally. The data suggest that the JNK pathway, through AP-1, plays no role in *Ddc* transcriptional induction *in vivo*.

2. A conserved NFκB site is also located upstream of *Ddc*, but is dispensable for its immune induction. Mutations in Toll and Imd pathway components do not affect *Ddc* induction following infection. Therefore, Toll and Imd signalling play no role in the induction of *Ddc* transcription in the innate immune response.

3. Flies bearing a mutation in *p38a* or *p38b* induced *Ddc* transcription normally. Therefore these mitogen-activated protein kinases (MAPKs) have no role in *Ddc* immune induction. However, the $p38c^{KG05834}$ flies failed to induce *Ddc* transcription following

septic injury, therefore the p38c MAPK pathway is responsible for induction of *Ddc* in the Drosophila immune response.

These results demonstrate a new role for *Ddc* in the Drosophila innate immune response. They show, for the first time, that the epidermis elicits a systemic response to septic injury. Furthermore, a new pathway in the immune defence repertoire of Drosophila has been identified. *Ddc* is likely induced in the epidermis to increase the amount of DA, which is then converted, by sclerotin processing enzymes, to cytotoxic reactive quinones that have an antimicrobial effect on invading bacteria.

Based on these results, several questions remain unanswered:

1. How is the p38c signal transduced?

The other p38 MAPKs, p38a and p38b, are both activated by the MAPK kinase (MAPKK), Licorne (Lic) (39,40). The MAPKK kinase (MAPKKK), MEKK1, is responsible for activation of Lic (40,41). Mutations in these kinases are homozygous lethal, and therefore, RNAi is the best mechanism to induce stage-specific knock-down of gene expression. Using this technique, I could deduce whether these kinases are important for p38c signalling and *Ddc* immune induction. A critical question is the identity of the transcription factor (TF) that acts through the AP-1 binding site I have shown is necessary for immune induction of *Ddc*. One TF, called activating transcription factor 2 (ATF2), has been shown to be activated by p38 MAPK signalling (42). Unfortunately, a mutant is not available; however, RNAi would facilitate characterization of a role for ATF2 in *Ddc* immune induction.

While it is possible that the previously identified members of the p38a and p38b MAPK families may transduce the p38c signal, the possibility also exists that novel MAPKKKs, MAPKKs, and TFs are involved. Identification of a putative MAPKK in the p38c MAPK pathway may aide in the understanding of how p38c is activated. This is particularly important given that the TGY dual phosphorylation motif has mutated to TDH in p38c (43). In addition, identification of potential TFs will add to our understanding of how p38c induces *Ddc* transcription. Following identification of putative TFs, I could characterize their ability to bind to *Ddc* and activate transcription.

To identify unique components of the p38c signalling cascade, I would employ the homologous RNAi library (44) to screen for modifiers of *Ddc* reporter gene induction in S2 cell culture. Optimization of a reporter gene would be essential. This may entail cloning the *Ddc* regulatory region upstream of the *hsp70* promoter to encourage higher basal expression of the gene. Double stranded RNAs that eliminate *Ddc* reporter gene immune induction represent activators of p38c signalling and could be further characterized. Likewise, precocious reporter gene expression could be induced when RNAi eliminates a pathway repressor. Using this scheme, one might identify signalling components upstream and downstream of p38c in the pathway. Thus, this scheme should allow dissection of the entire p38c MAPK pathway, including MAPKKKs, MAPKKs, and TFs.

2. What other genes are transcriptionally activated by p38c?

Since p38c induces the transcription of one gene responsible for the production of melanin and sclerotin in the Drosophila epidermis, it is possible it also induces the transcription of the other enzymes (32-34) that were identified to be immune inducible. Genome wide microarray analysis would be the most effective way to identify additional p38c response genes. This would involve comparing transcriptional induction of uninfected and infected wild-type and mutant flies. The genes that are induced following infection, in the wild-type, but not the *p38c* mutant flies, could be further characterized. Analysis of the promoter sequence of the p38c response genes will aide in the identification of putative p38c-activated TFs. Furthermore, identification of putative p38c response genes would provide potential alternative genes to use in the S2 cell RNAi screen outlined in the previous section if I was unable to optimize a *Ddc* reporter gene system.

3. Does p38c have kinase activity?

Since two amino acid substitutions in the catalytic domain have been proposed to eliminate the catalytic ability of this enzyme, determining whether it has kinase activity is essential. To do this, I would first purify p38c by *in vitro* expression. Then I would analyze its ability to phosphorylate the purified TFs that were identified in the RNAi cell culture screen described above.

References

1. McNabb, S.L., Baker, J.D., Agapite, J., Steller, H., et al. (1997). Disruption of a behavioral sequence by targeted death of peptidergic neurons in Drosophila. Neuron. 19: 813-823.
2. Park, J.H., Schroeder, A.J., Helfrich-Forster, C., Jackson, F.R., et al. (2003). Targeted ablation of CCAP neuropeptide-containing neurons of Drosophila causes specific defects in execution and circadian timing of ecdysis behavior. Development. 130: 2645-2656.
3. Dewey, E.M., McNabb, S.L., Ewer, J., Kuo, G.R., et al. (2004). Identification of the gene encoding *bursicon*, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr Biol. 14: 1208-1213.
4. Baker, J.D. and Truman, J.W. (2002). Mutations in the Drosophila glycoprotein hormone receptor, *rickets*, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J Exp Biol. 205: 2555-2565.
5. Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet. 24: 127-222.
6. Neckameyer, W.S. and White, K. (1993). *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. J Neurogenet. 8: 189-199.
7. Livingstone, M.S. and Tempel, B.L. (1983). Genetic dissection of monoamine neurotransmitter synthesis in Drosophila. Nature. 303: 67-70.
8. Hirsh, J. and Davidson, N. (1981). Isolation and characterization of the *Dopa decarboxylase* gene of *Drosophila melanogaster*. Mol Cell Biol. 1: 475-485.
9. Fraenkel, G. and Hsiao, C. (1962). Hormonal and nervous control of tanning in fly. Science. 138: 27-29.
10. Fraenkel, G., Hsiao, C. and Seligman, M. (1966). Properties of bursicon: An insect protein hormone that controls cuticular tanning. Science. 151: 91-93.
11. Mendive, F.M., Van Loy, T., Claeysen, S., Poels, J., et al. (2005). Drosophila molting neurohormone bursicon is a heterodimer and the natural agonist of the orphan receptor DLGR2. FEBS Lett. 579: 2171-2176.

12. Kimura, K., Kodama, A., Hayasaka, Y. and Ohta, T. (2004). Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*. Development. 131: 1597-1606.
13. Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., et al. (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila. J Neurosci. 26: 573-584.
14. Vie, A., Cigna, M., Toci, R. and Birman, S. (1999). Differential regulation of Drosophila tyrosine hydroxylase isoforms by dopamine binding and cAMP-dependent phosphorylation. J Biol Chem. 274: 16788-16795.
15. DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., et al. (1991). The Drosophila *Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics. 129: 385-397.
16. Hodgetts, R.B., Clark, W.C., O'Keefe, S.L., Schouls, M., et al. (1995). Hormonal induction of *Dopa decarboxylase* in the epidermis of Drosophila is mediated by the Broad-Complex. Development. 121: 3913-3922.
17. Chen, L., O'Keefe, S.L. and Hodgetts, R.B. (2002). Control of *Dopa decarboxylase* gene expression by the Broad-Complex during metamorphosis in Drosophila. Mech Dev. 119: 145-156.
18. Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W., et al. (2002). Induction of the early-late *Ddc* gene during Drosophila metamorphosis by the ecdysone receptor. Mech Dev. 114: 95-107.
19. Davis, M.M., Yang, P., Chen, L., O'Keefe, S.L., et al. (2007). The orphan nuclear receptor DHR38 influences transcription of the *Dopa decarboxylase* gene in epidermal and neural tissues of *Drosophila melanogaster*. Genome. 50: 1049-1060.
20. Kent, W.J. (2002). BLAT--the BLAST-like alignment tool. Genome Res. 12: 656-664.
21. Schug, J. (2003). Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence. In: Baxevanis, A.D. (ed.), Current Protocols in Bioinformatics. J. Wiley and Sons.

22. Bayer, C.A., Holley, B. and Fristrom, J.W. (1996). A switch in *broad-complex* zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of Drosophila imaginal discs. Dev Biol. 177: 1-14.
23. Emery, I.F., Bedian, V. and Guild, G.M. (1994). Differential expression of Broad-Complex transcription factors may forecast tissue-specific developmental fates during Drosophila metamorphosis. Development. 120: 3275-3287.
24. Lam, G. and Thummel, C.S. (2000). Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. Curr Biol. 10: 957-963.
25. Bender, M., Imam, F.B., Talbot, W.S., Ganetzky, B., et al. (1997). Drosophila *ecdysone receptor* mutations reveal functional differences among receptor isoforms. Cell. 91: 777-788.
26. Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E., et al. (2003). EcR isoforms in Drosophila: Testing tissue-specific requirements by targeted blockade and rescue. Development. 130: 271-284.
27. Wilhelm, J.E. and Smibert, C.A. (2005). Mechanisms of translational regulation in *Drosophila*. Biol Cell. 97: 235-252.
28. Enright, A.J., John, B., Gaul, U., Tuschl, T., et al. (2003). MicroRNA targets in *Drosophila*. Genome Biol. 5: R1.
29. Friggi-Grelin, F., Iche, M. and Birman, S. (2003). Tissue-specific developmental requirements of Drosophila tyrosine hydroxylase isoforms. Genesis. 35: 260-269.
30. Zhang, Y.Q., Friedman, D.B., Wang, Z., Woodruff, E., 3rd, et al. (2005). Protein expression profiling of the *Drosophila* fragile X mutant brain reveals up-regulation of monoamine synthesis. Mol Cell Proteom. 4: 278-290.
31. Denman, R.B., Dolzhanskaya, N. and Sung, Y.J. (2004). Regulating a translational regulator: Mechanisms cells use to control the activity of the fragile X mental retardation protein. Cell Mol Life Sci. 61: 1714-1728.
32. Boutros, M., Agaisse, H. and Perrimon, N. (2002). Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev Cell. 3: 711-722.

33. Irving, P., Troxler, L., Heuer, T.S., Belvin, M., et al. (2001). A genome-wide analysis of immune responses in Drosophila. Proc Natl Acad Sci USA. 98: 15119-15124.

34. De Gregorio, E., Spellman, P.T., Rubin, G.M. and Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA. 98: 12590-12595.

35. Mace, K.A., Pearson, J.C. and McGinnis, W. (2005). An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science. 308: 381-385.

36. Nappi, A.J., Carton, Y. and Vass, E. (1992). Reduced cellular immune competence of a temperature-sensitive *Dopa decarboxylase* mutant strain of *Drosophila melanogaster* against the parasite *Leptopilina boulardi*. Comp Biochem Physiol. 101: 453-460.

37. Silverman, N., Zhou, R., Erlich, R.L., Hunter, M., et al. (2003). Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J Biol Chem. 278: 48928-48934.

38. Vidal, S., Khush, R.S., Leulier, F., Tzou, P., et al. (2001). Mutations in the Drosophila *dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes Dev. 15: 1900-1912.

39. Suzanne, M., Irie, K., Glise, B., Agnes, F., et al. (1999). The Drosophila p38 MAPK pathway is required during oogenesis for egg asymmetric development. Genes Dev. 13: 1464-1474.

40. Zhuang, Z.H., Zhou, Y., Yu, M.C., Silverman, N., et al. (2006). Regulation of Drosophila p38 activation by specific MAP2 kinase and MAP3 kinase in response to different stimuli. Cell Signal. 18: 441-448.

41. Brun, S., Vidal, S., Spellman, P., Takahashi, K., et al. (2006). The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in Drosophila. Genes Cells. 11: 397-407.

42. Sano, Y., Akimaru, H., Okamura, T., Nagao, T., et al. (2005). Drosophila activating transcription factor-2 is involved in stress response via activation by p38, but not c-Jun NH(2)-terminal kinase. Mol Biol Cell. 16: 2934-2946.

43. Craig, C.R. (2003). *p38c* gene. Personal communication to FlyBase. http://flybase.bio.indiana.edu/reports/FBrf0182691.html

44. Foley, E. and O'Farrell, P.H. (2004). Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol. 2: E203.

Appendix 1: The regulation of tanning at pupariation

Cuticular tanning requires metabolites of dopamine (DA) (1). The first step in the synthesis of DA is the rate limiting conversion of tyrosine (Tyr) to dihydroxyphenylalanine (Dopa) by tyrosine hydroxylase (TH), which is encoded by the *pale* (*ple*) locus (2-4). The conversion of Dopa to DA is then catalyzed by Dopa decarboxylase (DDC) (encoded by *Ddc*) (5). In order for rapid tanning of the puparium to occur, large amounts of Tyr are required for DA synthesis. Tyr is a relatively insoluble compound and is stored as a more soluble derivative, tyrosine-*O*-phosphate (Tyr-P) late in the third larval instar (6). Tyr-P levels increase in third instar larvae to a maximum just prior to puparium formation. This is the only time that Tyr-P accumulates; no appreciable accumulation of Tyr occurs prior to eclosion (6,7). At pupariation, Tyr-P levels drop rapidly, with a corresponding increase in organic phosphate.

Tryptophan and phenylalanine hydroxylase (TPH), encoded by *henna* (*hn*), is a dual function enzyme with both tryptophan hydroxylase and phenylalanine hydroxylase (PAH) activity (8). It can convert tryptophan to 5-hydroxytryptophan, the precursor for serotonin synthesis, and phenylalanine to Tyr. This increases the amount of substrate available to TH for DA production. The PAH activity of TPH is likely central to the accumulation of large amounts of Tyr, in the form of Tyr-P, prior to pupariation. In fact, PAH activity increases throughout the third instar to a maximum at pupariation, and rapidly drops following puparium formation (7).

Both ecdysone, bound to its receptor, and the Broad (BR) Z2 isoform are required to bind regulatory sequences upstream of *Ddc* for proper expression at pupariation (9,10). Unfortunately, very little is known about the exact temporal expression of *ple* and *hn* at this time. To determine the precise timing of *ple, Ddc,* and *hn* transcription during the third instar, organisms were collected at the 2-3 moult and staged every 6 hours until puparium formation. Quantitative RT-PCR of both *ple* (Figure A1.1) and *Ddc* (Figure A1.2) epidermal transcripts reveals a small peak in transcription at the 2-3 moult, which drops to undetectable levels within 6 hours. Transcript levels of both genes remain low until they rise 3 hours prior to pupariation, which occurs approximately 63 hours after the 2-3 moult under our growth conditions. The rise in *Ddc* and *ple* transcription corresponds

to the onset of wandering behaviour. Levels of both transcripts reach a maximum at puparium formation. This suggests that both *ple* and *Ddc* transcripts accumulate, and are translated immediately, to produce the enzymes that are critical to the production of DA from Tyr for the tanning of the pupal case.

Tyr-P accumulates in the late third instar, suggesting that storage of Tyr as Tyr-P may be essential for the rapid synthesis of DA at pupariation (6). In order for storage of Tyr-P to occur, large amounts of Tyr need to be produced. Thus, it was not surprising when I found that *hn* transcript levels, which have a small peak at the 2-3 molt, return to basal levels within 6 hours and then rise dramatically 15 hours before puparium formation (Figure A1.3). Levels remain high until 3 hours prior to pupariation, after which they decline rapidly, reaching basal levels by puparium formation.

The early accumulation of *hn* transcripts, followed by a decline before pupariation (Figure A1.3), suggests that PAH activity is needed prior to pupariation to produce the Tyr that can then be converted to Tyr-P and stored. Tyr-P must be dephosphorylated to release the Tyr that is used for cuticular tanning. A phosphatase, capable of converting Tyr-P to Tyr, has been isolated from late third instar larvae (11). Furthermore, an epidermally expressed alkaline phosphatase (APH), that only accumulates during the late third instar, has been identified in *Drosophila* (12). While the exact relationship between these two phosphatases is unknown, it is likely that they are the same enzyme. Four different *Aph* genes, named *Aph*-1 through *Aph*-4, are encoded in the *Drosophila* genome (13-16). Based on enzyme electrophoretic variants, APH-1 was shown to be epidermally expressed in late third instar larvae and early pupae, and is therefore the best candidate for the APH that dephosphorylates Tyr-P (12,13,15). In addition, *in vitro* enzyme assays showed that Tyr-P was the best substrate for APH-1 (17,18). Thus, it is possible that Tyr is released from Tyr-P for tanning of the puparium through the actions of APH-1.

I have shown that the precise onset of post-eclosion cuticular tanning is regulated by the ecdysis neuropeptide cascade (See Chapter 2). This cascade does not play a role in regulation of tanning of the puparium, since these neuropeptides are not released until pupation, a full 12 hours after pupariation. Furthermore, unlike at eclosion, TH is not phosphorylated in white pre-pupae (data not shown), suggesting that activational

regulation of TH does not control the rapid tanning that occurs at pupariation. This suggests that DA synthesis at pupariation may be controlled by substrate availability rather than the translation and activation of TH that regulates adult cuticular tanning. Specifically, it appears that activation of APH-1, which leads to dephosphorylation of Tyr-P, may control DA synthesis at pupariation.

The *Aph*-1 gene is uncharacterized, although the gene has been mapped cytologically to between 75C1 and 85A6 on chromosome 3L (13). A BLAST search using the protein sequence of the only characterized *Drosophila* APH, APH-4 (14), located only one *Aph* gene in this region, at 78D2. This gene is predicted to contain four exons (Figure A1.4). A P-element insertion line (UM-8375-3) bearing a P{RS3} (19,20) element in the first intron of the putative *Aph-1* gene was obtained from Szeged Stock center. Homozygous UM-8375-3 flies are viable, with no obvious developmental defects, suggesting that this intronic insertion does not affect APH-1 function. I predicted that *Aph-1* null mutants would be late larval or early pupal lethal, since the inability to release Tyr from Tyr-P would cause a failure to tan the pupal case. However, functional redundancy in the *Aph* gene family could thwart my attempts to obtain an *Aph-1* mutant. Nevertheless, I set out to obtain an imprecise excision of the P-element, I was hoping to remove part of the *Aph-1* coding region and none of the sequence of the upstream or downstream genes and thereby produce a recessive lethal mutant. Since P{RS3} contains a mini-*white*$^+$ gene, I identified putative P-element excision mutants based on the presence of white eyes. I obtained 136 independent P-element excision lines, which I screened to determine if there was an excision of the P{RS3} element. I undertook a PCR based screen on isolated genomic DNA using primers anchored upstream and downstream of the P-element insertion site (Figure A1.4). Of the 136 excision mutants, 122 generated the ~120 bp wild-type PCR product. Of these lines, three were homozygous lethal (*Aph-1*^{3}, *Aph-1*^{25}, and *Aph-1*^{78}) (Figure A1.5). The fact that the wild-type band was the only band produced in these three lines suggests that the non-balancer chromosome contained a deletion of the *Aph-1* region. These lines were retained for further analysis. The 119 homozygous lines that generated a wild-type PCR product (See Figure 5, *Aph-1*^{78} and *Aph1*^{117} for an example) were discarded, since they contained a

precise excision, which would not disrupt APH-1 function. The remaining 14 lines did not have a wild-type copy of *Aph-1* (Figure A1.5). Of these, nine lines (*Aph-1*^{6}, *Aph-1*^{7}, *Aph-1*^{31}, *Aph-1*^{41}, *Aph-1*^{49}, *Aph-1*^{54}, *Aph-1*^{70}, *Aph-1*^{80} and *Aph-1*^{100}) generated PCR products that were larger than the wild-type band, indicating that these lines likely contained imprecisely excised P-elements that retained some of the P{RS3} sequence, or exogenous DNA that had been introduced due to repair from non-homologous template DNA. Since the P-element excisions were identified by reversion of the white^{+} phenotype, presumably the mini-*white*$^{+}$ gene was deleted or mutated in these lines. However, the sequence within each insertion was not determined. Southern analysis (performed by Lisa Chisholm-Dumesnil) confirmed that the nine lines contained an insertion in the *Aph-1* gene (data not shown). The final five lines (*Aph-1*^{55}, *Aph-1*^{57}, *Aph-1*^{94}, *Aph-1*^{96}, and *Aph-1*^{124}) did not generate a PCR product (Figure A1.5), suggesting that either the primer sites were missing or a product could not be formed, possibly due to a large insertion. The latter appears to be true, since Southern analysis (performed by Lisa Chisholm-Dumesnil) (data not shown) revealed that these lines contained large insertions in the intron of *Aph-1*. Presumably, I failed to obtain a PCR product in these lines due to the restrictions of the PCR program. Consistent with this prediction, I was unable to amplify the region that contained the P-element insertion in UM-8375-3 flies (Figure A1.5).

Since all of the homozygous viable lines tanned their puparium normally, we focused on characterizing the putative deletion mutants, *Aph-1*^{3}, *Aph-1*^{25}, and *Aph-1*^{78}, that were homozygous lethal. Southern analysis (performed by Sandra O'Keefe) (data not shown) revealed that *Aph-1*^{3} had a deletion extending into the downstream gene, CG5664 (Figure A1.4), so this line was discarded. It appears that both the upstream and downstream genes are intact in *Aph-1*^{25} and *Aph-1*^{78} (data not shown). Efforts are currently underway to clone and sequence the *Aph-1* region from these flies to determine the exact limits of the deletions. Following confirmation of a deletion in *Aph-1*, the mutant will be characterized. As a first step, Tyr-P production in the crude extracts from the heterozygotes bearing one chromosome with an *Aph-1* deletion could be compared to that in extracts from wild-type organisms.

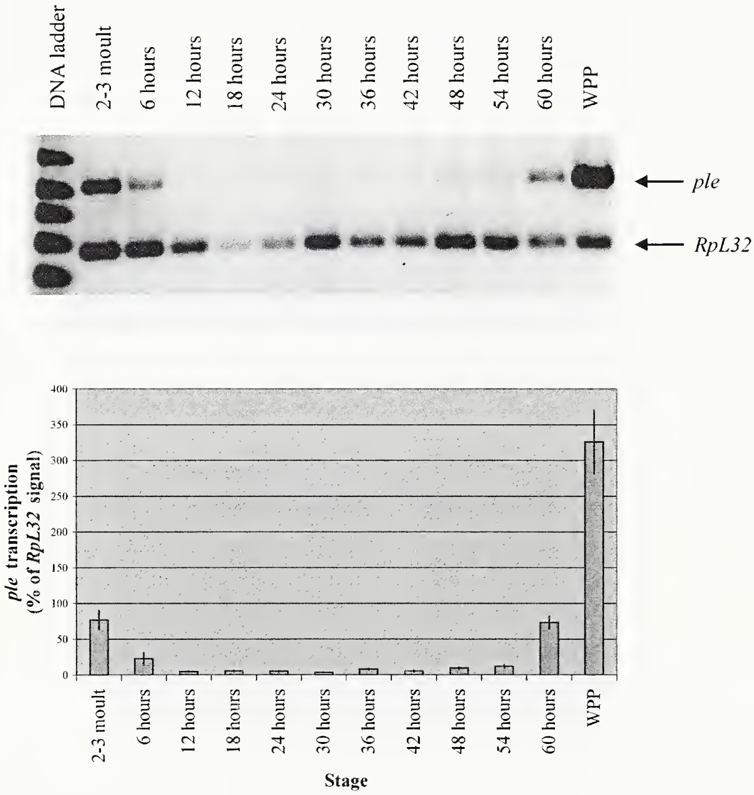


Figure A1.1: Quantitative RT-PCR of *ple* transcripts during the third instar. The *ple* transcripts are shown with an *RpL32* loading control. The time listed is the time after the 2-3 moult; white pre-pupae is abbreviated as WPP in this and subsequent figures. Bands from at least three different extracts for each time point were quantified and averaged, and expressed as a percent of the *RpL32* band to produce the graphs. Error bars in this and subsequent figures show standard error of the mean.

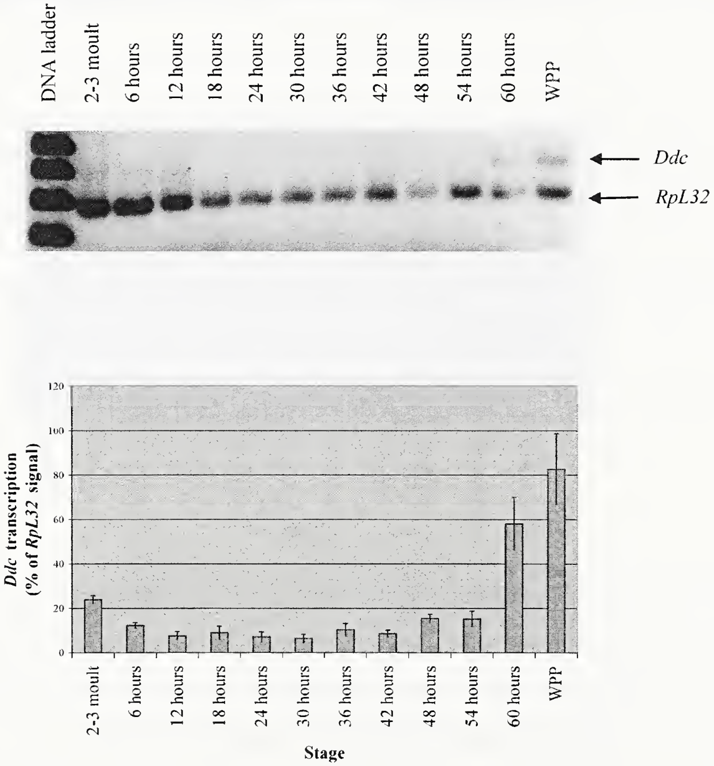


Figure A1.2: Quantitative RT-PCR of *Ddc* transcripts during the third instar. The *Ddc* transcripts are shown with an *RpL32* loading control. The time listed is the time after the 2-3 moult. Bands from at least five different extracts for each time point were quantified and averaged, and expressed as a percent of the *RpL32* band to produce the graphs.

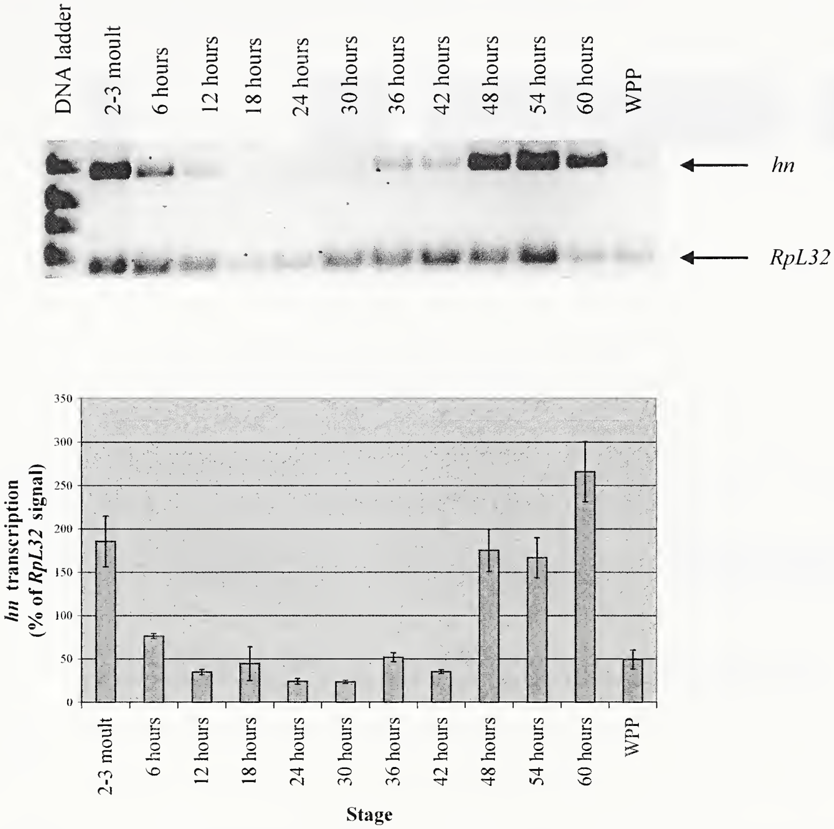


Figure A1.3: Quantitative RT-PCR of *hn* transcripts during the third instar. The *hn* transcripts are shown with an *RpL32* loading control. The time listed is the time after the 2-3 moult. Bands from at least four different extracts for each time point were quantified and averaged, and expressed as a percent of the *RpL32* band to produce the graphs.

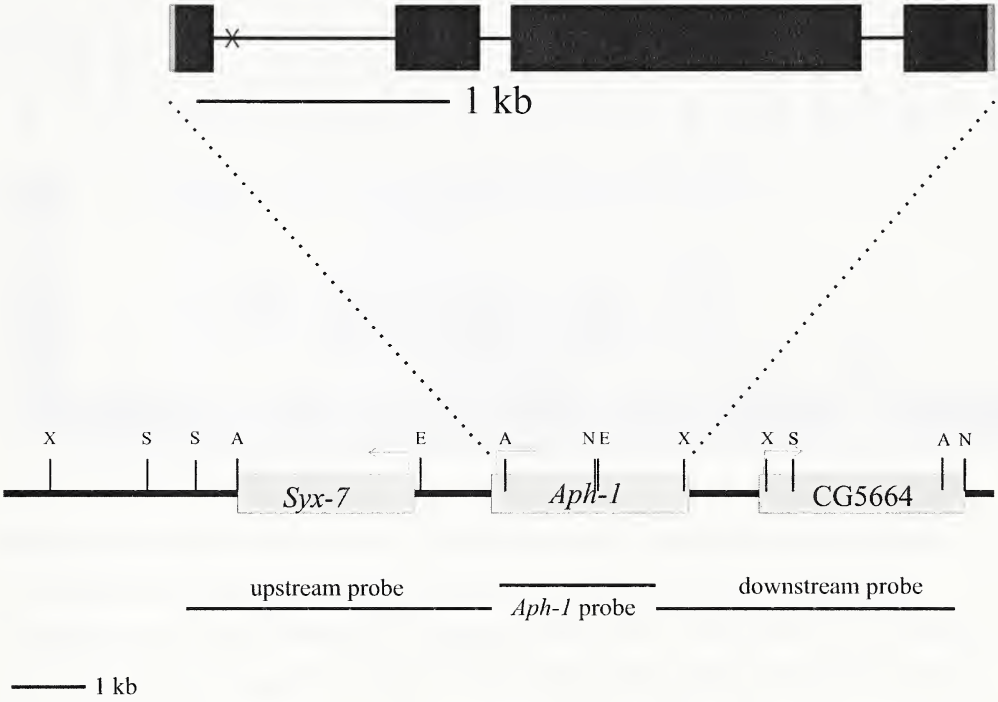


Figure A1.4: Schematic diagram of the putative *Aph-1* gene region. The 4 exons are shown (grey boxes). The coding sequence is shown in black. The X is the site of the P-element insertion in UM-8375-3. The PCR primers used in the PCR screen, APH-2F and APH-2R (See Appendix 2), flank this insertion site. *Aph-1* is sandwiched between two closely linked genes, *Syx-7* and CG5664. The direction of transcription of these genes is shown. The lines below the gene region represent the sequence used to make the probes use for Southern analysis. The restriction enzyme digestion sites used in Southern analysis are shown (E: *Eco*RV; X: *Xba*I; S: *Sma*I; N: *Nru*I).

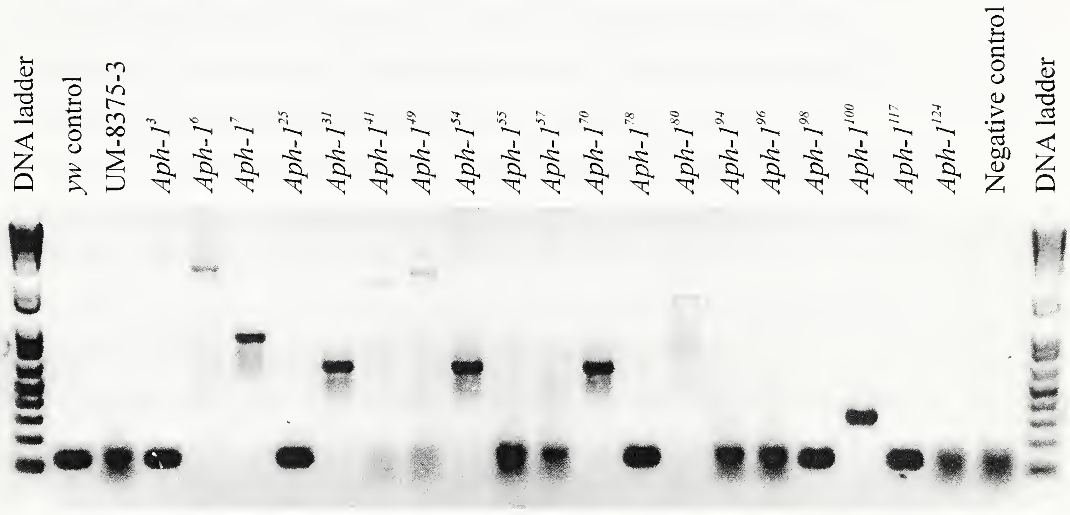


Figure A1.5: PCR analysis of 19 (of 136) putative *Aph-1* imprecise excision mutants. The wild-type PCR product is generated from DNA obtained from *yw* control flies amplified with primers flanking the P-element insertion site. This product is also seen in *Aph-1*^{3}, *Aph-1*^{25}, and *Aph-1*^{78}, which are balanced lines that contain one wild-type copy of *Aph-1* on the balancer *TM6, Ubx* chromosome, and *Aph-1*^{98}and *Aph-1*^{117}, which were eliminated from further analysis. *Aph-1*^{6}, *Aph-1*^{7}, *Aph-1*^{31}, *Aph-1*^{41}, *Aph-1*^{49}, *Aph-1*^{54}, *Aph-1*^{70}, *Aph-1*^{80} and *Aph-1*^{100} all appear to have an insertion in the first intron of *Aph-1*. No product was obtained from UM-8375-3, or *Aph-1*^{55}, *Aph-1*^{57}, *Aph-1*^{94}, *Aph-1*^{96}, and *Aph-1*^{124} (compare to negative control lane). The smeared band apparent in these lanes can be attributed to primer dimers.

References

1. Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv Genet. 24: 127-222.
2. Nagatsu, T., Levitt, M. and Udenfriend, S. (1964). Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem. 239: 2910-2917.
3. Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965). Elucidation of rate-limiting step in norepinephrine biosynthesis in perfused guinea-pig heart. J Pharmacol Exp Ther. 148: 1-8.
4. Neckameyer, W.S. and White, K. (1993). *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. J Neurogenet. 8: 189-199.
5. Livingstone, M.S. and Tempel, B.L. (1983). Genetic dissection of monoamine neurotransmitter synthesis in Drosophila. Nature. 303: 67-70.
6. Lunan, K.D. and Mitchell, H.K. (1969). The metabolism of tyrosine-*O*-phosphate in Drosophila. Arch Biochem Biophys. 132: 450-456.
7. Bel, Y., Jacobson, K.B. and Ferre, J. (1992). A comparative study of Drosophila phenylalanine hydroxylase with a natural and a synthetic tetrahydropterin as cofactor. Comp Biochem Physiol. 103: 557-562.
8. Neckameyer, W.S. and White, K. (1992). A single locus encodes both phenylalanine hydroxylase and tryptophan hydroxylase activities in Drosophila. J Biol Chem. 267: 4199-4206.
9. Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W., et al. (2002). Induction of the early-late *Ddc* gene during Drosophila metamorphosis by the ecdysone receptor. Mech Dev. 114: 95-107.
10. Chen, L., O'Keefe, S.L. and Hodgetts, R.B. (2002). Control of *Dopa decarboxylase* gene expression by the Broad-Complex during metamorphosis in Drosophila. Mech Dev. 119: 145-156.
11. Fukami, Y. and Lipmann, F. (1982). Purification of a specific reversible tyrosine-*O*-phosphate phosphatase. Proc Natl Acad Sci USA. 79: 4275-4279.
12. Schneiderman, H., Young, W.J. and Childs, B. (1966). Patterns of alkaline phosphatase in developing Drosophila. Science. 151: 461-463.

13. MacIntyre, R.J. (1966). Locus of the structural gene for 3rd larval instar alkaline phosphatase (Aph). DIS. 41: 61.

14. Yang, M.Y., Wang, Z., MacPherson, M., Dow, J.A., et al. (2000). A novel Drosophila alkaline phosphatase specific to the ellipsoid body of the adult brain and the lower Malpighian (renal) tubule. Genetics. 154: 285-297.

15. Parkash, R. and Vandna, V. (1994). Enzyme polymorphism in *D. melanogaster*. DIS. 75

16. Wallis, B.B. and Fox, A.S. (1968). Genetic and developmental relationships between two alkaline phosophatases in *Drosophila melanogaster*. Biochemical Genetics. 2: 141-158.

17. Harper, R.A. and Armstrong, F.B. (1973). Alkaline phosphatase of Drosophila melanogaster. II. Biochemical comparison among four allelic forms. Biochemical genetics. 10: 29-38.

18. Harper, R. and Armstrong, F.B. (1974). Alkaline phosphatase of *Drosophila melanogaster*. 3. Tyrosine-*O*-phosphate as substrate. Biochem Genet. 11: 177-180.

19. Golic, K.G. and Golic, M.M. (1996). Engineering the Drosophila genome: Chromosome rearrangements by design. Genetics. 144: 1693-1711.

20. Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., et al. (2004). The DrosDel collection: A set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. Genetics. 167: 797-813.

Appendix 2: Materials and Methods

Fly stocks and crosses (Chapter 2, 3 and Appendix 1)

The stocks used in this study, their source, and their purpose are shown in Table A2.1. All stocks were maintained on a standard cornmeal/molasses medium at 25°C in a 12 hour light-dark cycle, unless otherwise indicated. The mutation contained in the *burs*^{Z1091} allele causes a cysteine82 to tyrosine conversion and the *burs*^{Z5569} mutation results in a conversion of glycine115 to cysteine (1). The mutations in *rk*^{1} and *rk*^{4} flies introduce a premature stop codon in the transmembrane domain of this receptor (2). To obtain EH-KO flies, we crossed *w UAS-rpr* female virgins to *EH-Gal4* males. CCAP-KO flies have severe defects at the larval and pupal ecdyses, rarely yielding viable adult flies. In order to obtain a sufficient number of viable CCAP-KO adult flies for analysis, we crossed *yw; CCAP-Gal4* females to *w; UAS-rpr* males. In each experiment, *yw* flies were used as a wild-type control. To obtain *Ddc*^{ts2} *cn/ rdo hk Ddc*^{n7} *pr cn* and *Ddc*^{ts2}*cn/Df(2L)130 pr cn* flies, *Ddc*^{ts2} *cn/ CyO* female virgins were crossed to *rdo hk Ddc*^{n7} *pr cn/CyO* or *Df(2L)130 pr cn/CyO* males, respectively. The progeny were raised at the 18°C permissive temperature. Mutants were identified by their wild-type wings, and were separated from their curly-winged wild-type siblings, and transferred to the 25°C restrictive temperature for four days prior to infection.

To obtain the imprecise P-element excision mutants in *Aph-1* the following crosses were made:

w; Aph-1$^{UM\text{-}8375\text{-}3}$ ♀s X *w*/Y; Sb *e Δ2-3/TM6, Ubx e* ♂s
↓
yw; PSal89D, Sb/TM6, Ubx ♀s X *w*/Y; *Aph-1*$^{UM\text{-}8375\text{-}3}$*/Sb e Δ2-3* ♂s (red eyed)
↓
yw; PSal89D, Sb/TM6, Ubx ♀s X *yw*/Y; Aph-*1*/TM6, Ubx* ♂s
(* indicates a P-element excision, identified by the presence of white eyes)
↓
yw; Aph-1/TM6, Ubx* ♀s X *yw*/Y; *Aph-1*/TM6, Ubx* ♂s
↓
Homozygous or balanced stock

Neck ligations and CCAP and 8-Br-cAMP injections (Chapter 2)

Pupae were obtained from the side of a bottle and those that were within 6 hours of eclosion, according to previously defined cues (3), were mounted on double-sided tape on a slide, and the operculum was removed. As each fly emerged from the pupal case, it was immobilized on an ice-cooled Petri dish and was neck-ligated using a strand of hair. When injected, approximately 300 nL of 0.1 mM CCAP (Bachem) or 0.1M 8-Br-cAMP (Sigma) was delivered into the thorax through a fine glass capillary needle. Flies were kept in a humidified chamber until 2.5 hours after eclosion and frozen at -80°C for subsequent analysis.

Infection of organisms (Chapter 3)

All larvae that were infected were in the mid-third instar, during which time no epidermal *Ddc* transcription is detectible (See Appendix 1). Adults that were infected were between 4 and 6 days old, a time when epidermal *Ddc* transcripts are at minimal levels (See Chapter 2). At least 10 adults and 30 larvae of each genotype were septically injured with a sharpened tungsten needle that was dipped into the pellet obtained from a saturated culture of *E. coli* DH5α or *S. aureus* ATCC 6538-P. For heat shock experiments, organisms were incubated for 1 hour at 37°C, and allowed to recover at room temperature for 1 hour before being septically injured. Adults were injured by poking in the thorax immediately below the wing, and larvae were wounded in the posterior end. Adults were allowed to recover in a vial containing standard Drosophila medium and larvae recovered on a piece of sterile water-soaked Whatmann paper in a petri dish to prevent dessication after injury. With the exception of the time course shown in Figure 3.4, organisms were allowed to recover for 4 to 6 hours following infection. Only the organisms that were alive at harvesting were frozen for subsequent use.

RNA manipulations and RT-PCR (Chapters 2, 3 and Appendix 1)

RNA was extracted from pools of organisms using TRIzol, treated with amplification grade DNaseI, and reverse transcribed using Superscript II according to the manufacturer's instructions (Invitrogen). Table A2.2 shows the sequence of all of each primer used in these experiments. Synthesis of the *Ddc*, *ple*, *p38c*, *Ddc*-GFP, and *hn* specific cDNAs was initiated using the DDC1, PLE1, p38c-1, GFP1, and HN1 primers,

respectively, on 150 ng of isolated RNA. First strand reactions were co-reverse transcribed with RPL32-1, a primer specific for the ribosomal gene *RpL32*, which served as a loading control. A 3 μL aliquot of the resulting cDNA mixture was combined either with *ple* specific forward and reverse primers (PLE-F and PLE-R) or *hn* specific forward and reverse primers (HN-F and HN-R) and amplified for 5 cycles or with *Ddc* specific forward and reverse primers (DDC-F and DDC-R) or *Ddc*-GFP forward and reverse primers (DDC-F and GFP-R) and amplified for 3 cycles or *p38c* forward and reverse primers (p38c-2F and p38c-2R) and amplified for 6 cycles. The program was then stopped, *RpL32* gene specific primers (RPL32-F and RPL32-R) were added, and the program was allowed to continue for 23 more cycles. After an initial denaturing step of 2 minutes, the final PCR conditions for *ple* and *hn* consisted of 28 cycles of 95°C for 1 minute, 60°C for 1 minute and 73°C for 1 minute. The conditions were the same for *Ddc*, except the reaction ran for a total of 26 cycles.

Under these conditions, the amount of each product was proportional to input RNA concentration (Figure A2.1). Using 150 ng of input RNA, the amount of *Ddc* and *Ddc*-GFP product was proportional to number of cycles for 24-27 cycles, the amount of *ple* and *hn* product was proportional to the number of cycles for 26-29 cycles, the amount of *RpL32* product was proportional to the number of cycles for 22-25 cycles, and the amount of p38c product was proportional to the number of cycles for 27-30 cycles. The amount of DNA present in each band was quantified using Image J (4).

For non-quantitative RT-PCR (Figure 3.10B,C) on the *p38a* and *p38c* transcripts, greater than 1 μg of total RNA was reversed transcribed using Superscript II Reverse transcriptase (Invitrogen) using the primers p38a-R and p38c-1, respectively. The cDNA was then used as a template for a PCR reaction using the p38a-F and p38a-R primer pair for *p38a*, and the p38c-2F and p38c-2R primer pair for *p38c*. The final PCR reaction was: 30 cycles of: 95°C for 1 minute, 60°C for 1 minute, and 73°C for 1 minute.

Western blots (Chapter 2)

Organisms were macerated in running buffer (0.125 M Tris-HCl pH 6.8, 12.5% glycerol, 2.5% SDS, 1.25% β-mercaptoethanol, and 0.025% bromophenol blue), boiled for 10 minutes, and 0.5 (TH detection), 1 (phospho-Ser32 TH detection), or 0.25 (actin detection) organism equivalents were loaded and separated on 8% denaturing acrylamide

gels. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Hybond-ECL). Membranes were blocked for 1 hour in Tris-buffered saline with 0.1% Tween (TBS-T) + 1% skim milk powder. Membranes were then incubated with either rabbit anti-TH antibody (1:3000 dilution, 3 hours at room temperature (RT)), rabbit anti-phospho-TH (Ser40) (1:2000 dilution, overnight at 4°C), or mouse anti-actin (1:10000 dilution, 1 hour at RT). The rabbit anti-TH antibody was a gift from Wendy Neckameyer, the rabbit anti-phospho-TH (Ser40) antibody was purchased from Zymed Laboratories, and the mouse anti-actin (JLA-20) antibody developed by Jim Jung-Ching Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. The membranes were then washed with TBS-T and incubated with either a goat anti-rabbit IgG (H + L)-HRP Conjugate (Bio Rad) or an ECL anti-rabbit IgG HRP-linked species-specific whole antibody (from donkey) (Amersham Biosciences), or a goat anti-mouse IgG (H+L)-HRP conjugate (Invitrogen) (1:1000 dilution, 1 hour at RT). Membranes were then washed with TBS-T and developed using Supersignal West Pico Chemiluminescent Substrate (Pierce) according to manufacturer's instructions.

Tyrosine hydroxylase activity assays (Chapter 2)

Except where indicated, all reagents were obtained from Sigma. Pools of organisms were macerated in 1X phosphate buffered saline containing 1X Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche) (25μL per organism). The extract was cleared by centrifugation, and a 25 μL aliquot of the supernatant was added to the reaction mixture (50 mM PIPES, pH 8.0, 25 μM L-tyrosine, 5 mM DTT, 10 μM $Fe(NH_4)_2(SO_4)_2$, 40 μg/ml catalase and 1 μCi L-[3,5^{3}H]-tyrosine (Amersham), in a total volume of 49 μL. The reaction mixture was equilibrated to 27°C for 3 minutes, and then 1 μL of 100 μM tetrahydrobiopterin or H_2O (for blanks) was added to start the reaction. The reaction was incubated at 27°C for 15 minutes. Residual L-[3,5]^{3}H-tyrosine was removed with 500 μL of 7.5% activated charcoal in 1M HCl. 100 μL of the supernatant was combined with 10 mL of scintillation fluor and counted for 1 minute.

Plasmid construction and the creation of transgenic lines (Chapters 2, 3 and Appendix 1)

The P-element plasmids P[*Ddc*-GFP]PH, P[*Ddc*-GFP]SH, P[*Ddc*-GFP]BH, and P[*Ddc*-GFP]EH (Figure 1) have been described elsewhere (5). The P[*Ddc*]PHΔBE, P[*Ddc*]PHΔBE1, P[*Ddc*]PHΔBE2, P[*Ddc*]PHΔBE3, P[*Ddc*]PHmutNFκB, and P[*Ddc*]PHmutAP-1 in pBluescript SK(+) constructs were all created by inverse PCR using phosphorylated primer pairs (See Table A2.2) ΔBE3-F and ΔBE1-R, ΔBE1-F and ΔBE1-R, ΔBE2-F and ΔBE2-R, ΔBE3-F and ΔBE3-R, NFκB-F and NFκB-R, and AP1-F and AP1-R respectively, on PH in pBluescriptSK(+). The resulting products were gel purified, and intramolecularly ligated to create the respective plasmids. The breakpoints of the deletions and site-specific mutations were all confirmed by sequencing. The *Ddc* region contained within each plasmid was then liberated by digestion with *Bam*HI and *Kpn*I and cloned into similarly digested pGreen Pelican (6) to create P[*Ddc*-GFP]PHΔBE, P[*Ddc*-GFP]BE-1, P[*Ddc*-GFP]BE-2, P[*Ddc*-GFP]BE-3, P[*Ddc*-GFP]mutNFκB, and P[*Ddc*-GFP]mutAP-1.

To create the P[*Ddc*-RNAi]pSymp and P[*Ddc*-RNAi]pWIZ constructs, a 490 base pair region of the *Ddc* coding sequence within the 4th exon was amplified with the DDC-*Not*IF and DDC-*Not*IR and DDC-*Xba*IF and DDC-*Xba*IR primer pairs. The DDC-*Not*I primers add *Not*I sites to facilitate cloning into pSymp (7), and the DDC-*Xba*I primers add *Xba*I sites to facilitate cloning into pWIZ (8). Both pSymp and pWIZ express the cloned region under the control of a UAS promoter, facilitating Gal4 induction of the transgene. The PCR fragments were gel purified and cloned into pGemT-easy (Promega) to create *Ddc-Not*I in pGemT-easy and *Ddc-Xba*I in pGemT-easy. The *Ddc* fragment was then liberated from *Ddc-Not*I in pGemT-easy by *Not*I digestion and cloned into similarly digested pSymp to create P[*Ddc*-RNAi]pSymp. To create P[*Ddc*-RNAi]pWIZ, the *Xba*I fragment from *Ddc-Xba*I in pGemT-easy was first cloned into the *Avr*II site of pWIZ, and then cloned into the *Nde*I site of the resulting plasmid. Both plasmids were sequenced to confirm the orientation of the *Ddc* fragment inserted into each site.

DNA solutions (0.4 μg/μL DNA in 0.5X phosphate buffered saline and 10% glycerol) containing transgenic constructs were injected into embryos of the genotype: *w*; *Sb*Δ*2*-

3/TM6, Ubx. Surviving adults were crossed to *yw* flies and progeny with the w^+ phenotype were collected and mated to produce homozygous or balanced stocks.

To create the plasmids that were used for Southern probe preparation (Appendix 1), genomic DNA was isolated (9) from *yw* control flies and was first amplified with Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The region upstream of *Aph-1* (3995 bp), the *Aph-1* region (2053 bp), and the region downstream of *Aph-1* (3907 bp) were amplified with the APH-US-F and APH-US-R, the APH-1F and APH-1R, and the APH-DS-F and APH-DS-R primer pairs, respectively. The PCR products were gel purified and cloned into pGem-T-easy (Promega). The insert sequence was confirmed by sequencing.

Production of recombinant proteins (Chapter 3)

The dFRA (Drosophila *fos*) and dJRA (Drosophila *jun*) in pBluescript KS(+) constructs were a generous gift from Robert Tjian. These plasmids were used as templates for *in vitro* transcription and translation using the TNT T3 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assays (Chapter 3)

Mobility shift assays were carried out using a 26 base pair double-stranded oligonucleotide probe with a 2 base pair extension. The 28 base single-stranded oligonucleotides were individually end-labelled with γ-^{32}P-ATP using T4 kinase (Invitrogen) according to the manufacturer's instructions, and annealed. The wild-type probe was obtained by annealing the primer pair AP1-A and AP1-B (the putative AP-1 site is underlined and bold in Table A2.2), and the probe containing the mutated AP-1 site (mutated bases are uppercase) was obtained by annealing AP1M-A and AP1M-B.

The recombinant proteins or reticulocyte lysate mix were incubated in binding buffer (20 mM Hepes-KOH pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM DTT, 0.05% NP-40 and 20% glycerol) in the presence of 2 μg of the non-specific competitor poly(dI-dC) (Amersham) and specific cold competitor, where applicable, for 5 minutes at room temperature. Two ng of radiolabelled probe was added to the reaction and allowed to bind at room temperature for 15 minutes. Binding mixtures were fractionated on a 6% polyacrylamide gel in 0.5X TBE running buffer and the products were visualized by autoradiography.

PCR screening of putative P-element excision lines (Appendix 1)

PCR screening was carried out on isolated genomic DNA (9) and amplified using APH-2F and APH-2R, which are anchored on either side of the P-element insertion. Lines that generated the ~100 bp wild-type product, representing a precise excision, were eliminated from further analysis.

Southern Analysis (Appendix 1)

Genomic DNA was extracted (9) from the putative *Aph-1* mutant lines, and three sets of double digests were done: 1. *Eco*RV and *Xba*I; 2. *Sma*I and *Apa*I; and *Nru*I and *Xba*I. Ten µg of DNA, per lane, were run on a 1% agarose gel and transferred overnight by capillary action to a Biodyne B nylon membrane (Pall). Using the plasmids described above as template, the DECAprime Kit (Ambion) was employed to make ^{32}P-labelled dCTP probes according to manufacturer's instructions, for either the upstream, *Aph-1*, or downstream region. Prehybridization and hybridization were carried out at 65°C in Church's Buffer (1 mM EDTA, 0.5 M $NaPO_4$ pH 7.2, 7% SDS) with sheared salmon sperm DNA. The membrane was then washed once with 2X SSC, 0.5% SDS at room temperature, twice with 0.2X SSC, 0.1% SDS at room temperature and twice with 0.1X, 0.1 % SDS at 65°C. Bands were then visualized by autoradiography and sized using a standard curve made with a 1 kb Plus DNA ladder (Invitrogen).

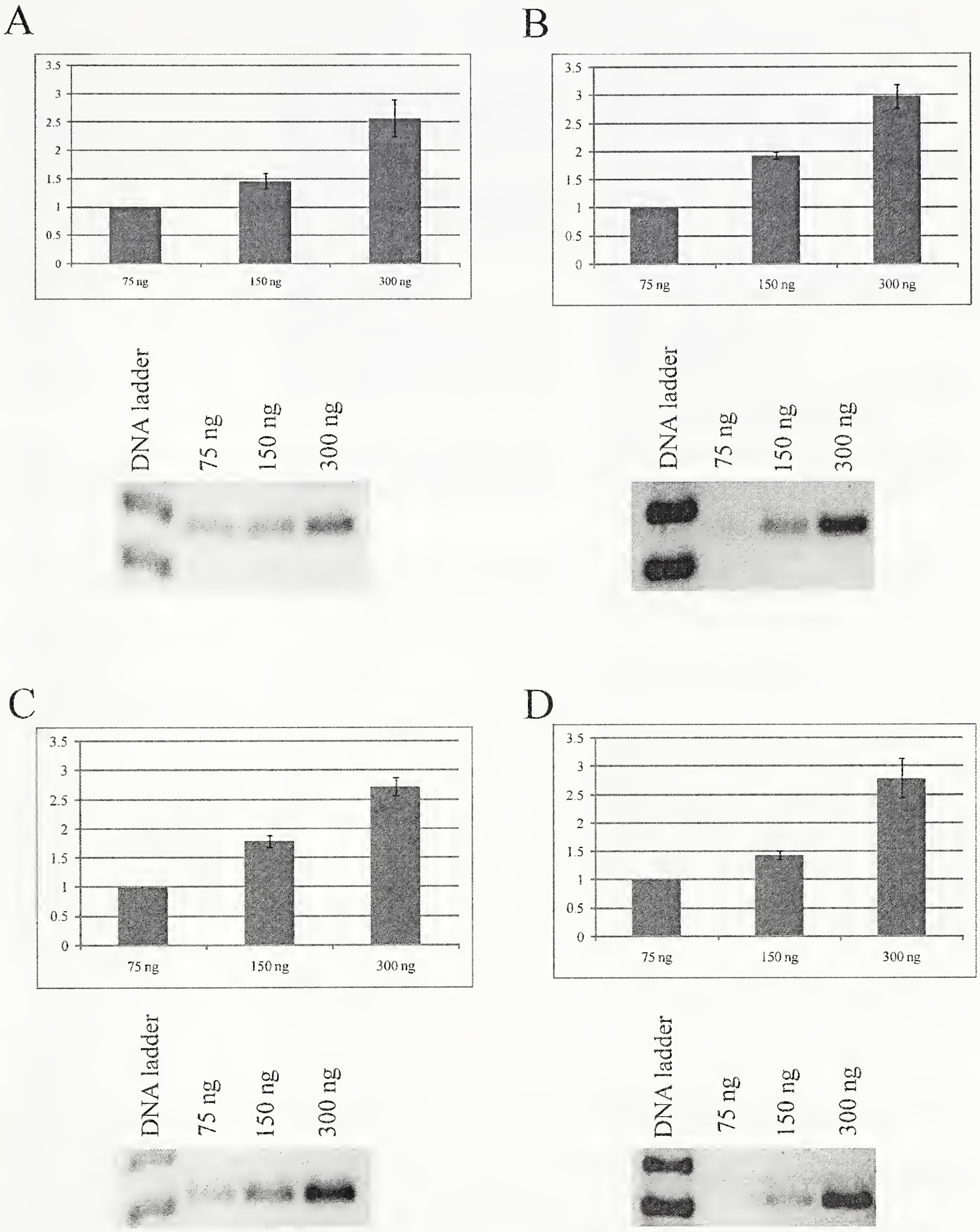


Figure A2.1: PCR product accumulation is proportional to amount of input RNA. A. *Ddc*-specific; B. *RpL32*-specific; C. *Ddc*-GFP-specific; and D. *p38c*-specific semi-quantitative RT-PCR with 75, 150, and 300 ng of input RNA.

Genotype	**Source**	**Use/nature of allele**
yw	Bloomington stock #1495	wild-type control
EH-Gal4	Bloomington stock #6301	Gal4 expression in EH neurons
CCAP-Gal4	Douglas Allen	Gal4 expression in CCAP neurons
w UAS-*rpr*	Bloomington stock #5823	expression of *rpr*
w; UAS-*rpr*	Bloomington stock #5824	expression of *rpr*
burs^{Z1091}	Susan McNabb	hypomorphic mutation in *burs*
burs^{Z5569}	Susan McNabb	hypomorphic mutation in *burs*
rk^{4}	Bloomington stock #3590	null mutation in *rk*
rk^{1}	Bloomington stock #3589	null mutation in *rk*
dom^{K08108}	Bloomington stock #10767	P-element insertion in *dom*, which causes loss of haemocytes
Ddc^{ts2} *cn/CyO*	Bloomington stock #3004	temperature sensitive *Ddc* mutant
Df(2L)130 pr cn/CyO	Bloomington stock #2349	Deletion removes the *Ddc* gene
rdo hk Ddc^{n7} *pr cn/CyO*	(10)	Null allele of *Ddc*
Act5C-Gal4	Bloomington stock #3954	Actin promoter expressing Gal4
yw; P[*Ddc*-RNAi]pWIZ	This study	*Ddc* RNAi construct
yw; P[*Ddc*-RNAi]pSymp	This study	*Ddc* RNAi construct
w;hs-Gal4	Bloomington stock #1799	heat shock promoter expressing Gal4
yw; P[*Ddc*-GFP]PH	(5)	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]SH	(5)	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]BH	(5)	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]EH	(5)	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHΔBE	This study	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHΔBE1	This study	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHΔBE2	This study	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHΔBE3	This study	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHmutNFκB	This study	*Ddc* reporter construct bearing line
yw; P[*Ddc*-GFP]PHmutAP-1	This study	*Ddc* reporter construct bearing line
Act5C-Gal4	Bloomington stock #3954	Actin promoter expressing Gal4
w;hs-Gal4	Bloomington stock #1799	heat shock promoter expressing Gal4
UAS-*hep*	Bloomington stock #6406, #9307, and #9308	expression of a wild-type HEP

UAS-*bsk*	Bloomington stock #6407 and #9310	expression of a wild-type BSK
UAS-*jun*	Bloomington stock #7216	expression of a wild-type Jun
UAS-*fos*	Bloomington stock #7213	expression of a wild-type Fos
UAS-*hepACT*	Bloomington stock #9305 and #9306	expression of a constitutively active HEP
UAS-*bskDN*	Bloomington stock #6409 and #9311	expression of a dominant negative BSK
UAS-*junDN*	Bloomington stock #7217 and #7218	expression of a dominant negative Jun
UAS-*fosDN*	Bloomington stock #7214 and #7215	expression of a dominant negative Fos
yw; Tak1	Edan Foley	Imd and JNK pathway deficient
yw; ird5^{KG08072}	Bloomington stock #14684	P-element insertion in the first exon of *ird5*
yw; ird5^{EY02434}	Bloomington stock #19825	P-element insertion in the first exon of *ird5*
yw; Rel^{EY08061}	Bloomington stock #20016	P-element insertion in the first exon of *Rel*
mwh^{1} *kni*$^{ri-1}$ *snk*^{4} *red*^{1} *e Tl*^{3} *ca/Ms(3) R24/TM3, Sb*	Bloomington stock #3338	Balanced gain of function *Tl* mutant
rl^{1}	Bloomington stock #386	spontaneous hypomorphic mutation in ERK
Mpk2^{1}	Bloomington stock #8822	deletion of *p38a* MAPK gene
p38b^{KG02737}	Bloomington stock #12905	P-element insertion in 5' region of the *p38b* MAPK gene
p38b^{KG01337}	Bloomington stock #14364	P-element insertion in 5' region of the *p38b* MAPK gene
yw; p38c^{KG05834}	Bloomington stock #14126	P-element insertion in the coding sequence of the *p38c* MAPK gene
w; Aph-1$^{UM-8375-3}$	Szeged stock #UM-8375-3	P-element insertion in the first intron of the *Aph-1* gene
w/Y; Sb e Δ2-3/TM6, Ubx e	James MacLagan	Source of constitutive transposase expression
yw; PSal89D, Sb/TM6, Ubx	John Locke	Source of the balancer chromosome *TM6, Ubx*

Table A2.1: Fly stocks used in this thesis. Where applicable, the source of the stock and the nature of the mutation or the purpose of the stock are given. *CyO = In (2LR)O, Cy dp*^{lvI} *pr cn*^{2}

Primer name	Sequence (5'-3')	Orientation
PLE-1	cgggctcgtacttggtcatc	Reverse
PLE-F	tccctccgagcaagatgtgg	Forward
PLE-R	accacggagccttgacattc	Reverse
DDC-1	aatcgctcactcagcatgt	Reverse
DDC-F	gccaagcgcacagcaatcag	Forward
DDC-R	atgactcgctcgatgtcctg	Reverse
RPL32-1	cttcttgagacgcaggcga	Reverse
RPL32-F	agcatacaggcccaagatcg	Forward
RPL32-R	agtaaacgcgggttctgcat	Reverse
GFP-1	tgtggcggatcttgaagttc	Reverse
GFP-2R	ggcgcgggtcttgtagttgc	Reverse
ΔBE1-F	tagccgagccgatataccca	Forward
ΔBE1-R	aagtattctccgcttatagg	Reverse
ΔBE2-F	cccgctcaaaaggctcaacc	Forward
ΔBE2-R	tgggtatatcggctcggcta	Reverse
ΔBE3-F	gggaagcacgtgagcagaat	Forward
ΔBE3-R	ggttgagccttttgagcggg	Reverse
NFκB-F	cctgacgagatcgctctctttc	Forward
NFκB-R	aGAGAGccaattatgagtcactcgaaaca	Reverse
AP1-F	ctcgaaacagggtttttcttgg	Forward
AP1-R	GCagATGtaattgggggattcctgacg	Reverse
p38a-F	tggaaaagatgttgag	Forward
p38a-R	tatcctcgaagctgtgatcg	Reverse
p38c-1	gcggtgtcttcggcatgatg	Reverse
p38c-2F	caccagcgcacaacatgatg	Forward
p38c-2R	tcgcacctttgtcgtaacgg	Reverse
AP1-A	ccctgtttcgag**tgactca**tattggggg	Forward
AP1-B	taccccccaata**tgagtca**ctcgaaacag	Reverse
AP1M-A	ccctgtttcgagGCacATGtattggggg	Forward
AP1M-B	taccccccaataCATgtGCctcgaaacag	Reverse
DDC-*Not*IF	*gcggccgcgcggccgc*gagccaaggccaagaagtt	Forward
DDC-*Not*IR	*gcggccgcgcggccgc*cacccaactgggatccttc	Reverse
DDC-*Xba*IF	*tctagatctaga*gagccaaggccaagaagtt	Forward
DDC-*Xba*IR	*tctagatctaga*cacccaactgggatccttc	Reverse
HN-1	cctctcggaatccacagttg	Reverse
HN-2F	gttcgccatcggttttattg	Forward
HN-2R	tgccccaggtctcgatctcc	Reverse
APH-US-F	gaaccacagggcagagatcc	Forward
APH-US-R	cggggcgttacgaagtctac	Reverse
APH-F1	cccaactcacggcctgtagc	Forward
APH-R1	cgtgtgtctcctcggtcatgg	Reverse
APH-DS-F	ccatgaccgaggagacacacg	Forward

APH-DS-R	aaacggtaacgaatcaaatgca	Reverse
APH-F2	tccaactcacggcctgtagc	Forward
APH-R2	cgacctggcacactctcact	Reverse

Table A2.2: PCR primers and oligonucleotide probes that were used in this thesis. The mutated bases in the respective AP-1 and NFκB binding sites are shown in capital letters. The AP-1 binding site in the probe used in the mobility shift experiment is shown in bold and underlined. The *Not*I sites and the *Xba*I sites in the DDC-*Not*I and DDC-*Xba*I primers pairs, respectively, are shown in italics.

References

1. Dewey, E.M., McNabb, S.L., Ewer, J., Kuo, G.R., et al. (2004). Identification of the gene encoding *bursicon*, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr Biol. 14: 1208-1213.
2. Baker, J.D. and Truman, J.W. (2002). Mutations in the *Drosophila* glycoprotein hormone receptor, *rickets*, eliminate neuropeptide-induced tanning and selectively block a stereotyped behavioral program. J Exp Biol. 205: 2555-2565.
3. Kimura, K. and Truman, J.W. (1990). Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster*. J Neurosci. 10: 403-411.
4. Abramoff, M.D., Magelhaes, P.J. and Ram, S.J. (2004). Image Processing with Image J. Biophotonics International. 11: 36-42.
5. Davis, M.M., Yang, P., Chen, L., O'Keefe, S.L., et al. (2007). The orphan nuclear receptor DHR38 influences transcription of the *Dopa decarboxylase* gene in epidermal and neural tissues of *Drosophila melanogaster*. Genome. In press
6. Barolo, S., Carver, L.A. and Posakony, J.W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. BioTechniques. 29: 726-732.
7. Giordano, E., Rendina, R., Peluso, I. and Furia, M. (2002). RNAi triggered by symmetrically transcribed transgenes in *Drosophila melanogaster*. Genetics. 160: 637-648.
8. Lee, Y.S. and Carthew, R.W. (2003). Making a better RNAi vector for Drosophila: Use of intron spacers. Methods. 30: 322-329.
9. Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16: 1215.
10. Wright, T.R., Steward, R., Bentley, K.W. and Adler, P.N. (1981). The Genetics of *Dopa decarboxylase* in *Drosophila melanogaster* III. Effect of a temperature sensitive Dopa decarboxylase Deficient mutation of female fertility. Dev Genet. 2: 223-235.